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Tesi di Dottorato

THE ROLE OF BIOMARKERS IN CARDIOVASCULAR EMERGENCY. A CLINICAL STUDY ON A NOVEL BIOCHEMICAL METHOD FOR THE DIAGNOSIS OF AORTIC DISSECTION.

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ABBREVIATIONS

CVD = cardiovascular disease

MI = myocardial infarction

ECG = electrocardiogram

cTnT = cardiac troponin T

cTnI = cardiac troponin I

CK-MB = creatine kinase-MB

ACS = acute coronary syndrome

AD = aortic dissection

AAS = acute aortic syndrome

CT = computerized tomography

MRI = magnetic resonance imaging

TEE = transesophageal echocardiography

TTE = transthoracic echocardiography

SMMHC = smooth muscle myosin heavy chain

DD = D-dimer

sELAF = soluble elastin fragments

MMPs = matrix metalloproteinases

ABSTRACT

Background: Aortic dissection (AD) is a life-threatening medical emergency of the aorta, characterized by disruption of the aortic media by blood entering through a laceration of the luminal vascular wall. Therefore, rapid diagnosis and timely management play an essential role in patient survival. Although newer diagnostic methods have greatly improved the diagnosis of AD, the diagnosis is still frequently missed today because the clinical manifestations of AD are often diverse and the clinical presentation may mimic signs and symptoms of other diseases. A reliable biochemical diagnostic method for AD would be beneficial.

Objectives: to analyze the main demographic characteristics (sex, age) and a common symptom of cardiovascular emergency, as chest pain, in patients with suspected or confirmed AD; to enstablish the utility of the troponin- like protein of smooth muscle, calponin, as a diagnostic biomarker of AD.

Methods: From April 2004 to June 2007, the patients with suspected AD were enrolled in the multicenter study. Clinical data forms were completed for each of the patients. Blood plasma was drawn on admission and used for measurements. Finally, an immunoassay against circulating calponins was generated by Biosite Incorporated.

Results: In Italy, 412 patients (62,8 ± 13,4 years) have been enrolled including 151 (36,7%) with AD (60,9 ± 13,4 years) and 261 (63,3%) with a different final diagnosis (63,8 ± 12,7 years). The chest pain was the most common symptom (77,4%): the half of patients (50,5%) had severe pain and referred that the chest pain had a abrupt onset (54,2%).

From all enrolled patients into the international study, the plasma specimens of 217 patients have been analysed including 59 cases of AD and 158 cases with an initial suspicion of AD but a different final diagnosis. Basic and acidic calponins, respectively, showed greater than two-fold and three-fold elevations in patients with AD.

Conclusions: The descriptive analysis of data shows that chest pain was the most common symptom in cases of AD but, given the relative frequency of chest pain in patients presenting to emergency room and the relative infrequency of AD, the availability of biomarkers could be of great assistance in carrying out the differential diagnosis between the diseases that are accompanied by chest pain.

Acid and basic calponins have the potential for use as an early diagnostic biomarker for AD but the results of this preliminary experience using an initial assay show moderate sensitivities and specificities with negative predictive values which should be further improved upon.

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death in most western societies and is increasing steadily in many developing countries (1). Thus, primary prevention and secondary prevention of CVD are public health priorities (2).

Great progress has been made in the past quarter century in the diagnostic biochemical testing of CVD. Beginning with the introduction of the serum transaminase assays (3), followed by clinical application of enzyme activity assays (e.g., lactate dehydrogenase, creatinine kinase) which greatly improved the diagnosis of acute myocardial infarction (4-5), and recently assays of structural proteins (e.g., cardiac myosin light chain and troponin) (6-7) have established their roles in clinical medicine.

Clinicians have used additional tools to facilitate clinical assessment and to enhance their ability to identify the patient at risk for CVD.

Biomarkers are one such tool to better identify high-risk individuals, to diagnose disease conditions promptly and accurately, and to effectively prognosticate and treat patients with disease (8).

Biomarkers, defined as alterations in the constituents of tissues or body fluids, provide a powerful approach to understanding the spectrum of CVD with applications in at least 5 areas: screening, diagnosis, prognostic, prediction of disease recurrence, and therapeutic monitoring (9).

With expansion of the number and types of existing biomarkers, the opportunity to improve diagnosis, risk stratification, and selection of therapy using these non-invasive, affordable tools continues to grow. Congruent with this evolution, the practicing clinician will benefit from a thorough understanding of the clinical, biology and technology evidence underlying the use of established and emerging biomarkers in CVD (10).

The research doctorate in physiopathology of cardiovascular apparatus has represented an important opportunity not only to acquire a better knowledge in the field of cardiology, but also to propose an innovative experience in the use of biological markers for the early diagnosis of CVD and in particular of the aortic dissection (AD).

This study has been funded by Biosite Incorporated, a pharmaceutical firm involved in biomarkers discovery, and it has been carried out together with a research team of ISBEM ScpA (Scientific Biomedical Euro Mediterraneam Institute) from Brindisi.

1. THE BIOMARKERS

The term biomarker (biological marker) was introduced in 1989 as a Medical Subject Heading (MeSH) term: "measurable and quantifiable biological parameters which serve as indices for health- and physiology-related assessments".

In 2001, an NIH (National Institutes of Health) working group standardized the definition of a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (11).

A biomarker may be measured on a biosample (as a blood, urine, or tissue test), it may be a recording obtained from a person (blood pressure, electrocardiogram, or Holter), or it may be an imaging test (echocardiogram or computerized tomography scan).

A simplistic way to think of biomarkers is as indicators of disease trait (risk factor or risk marker), disease state (preclinical or clinical), or disease rate (progression) (12).

Accordingly, biomarkers can be classified as antecedent biomarkers (identifying the risk of developing an illness), screening biomarkers (screening for subclinical disease), diagnostic biomarkers (recognizing overt disease), staging biomarkers (categorizing disease severity), or prognostic biomarkers (predicting future disease course, including recurrence and response to therapy, and monitoring efficacy of therapy) (11).

Regardless the purpose for its use, a new biomarker will be clinical valuable only if it is accurate, it is reproducibly, obtained in a standardized fashion, it is acceptable to the patient, it is easy to interpret by clinicians, it has high sensitivity and high specificity for the outcome which is expected to identify (13).

Table 1 displays the desirable properties of biomarkers overall and of biomarkers of screening, diagnosis, and prognosis (14).

Although several biomarkers satisfy one or more of these criteria, no single marker has been identified yet that satisfies all of them.

Today, in the whole world, there are investigators researching new biomarkers. The entire process, from the discovery of a biomarker in a laboratory, to the development of an assay and finally to its delivery, requires many years (15) [Figure 1]. Briefly, the process begins with the identification of target biomarkers with the use of standardized technology platforms, followed by validation of the assays (16, 17), statistical evaluation of biomarker distributions in reference samples and in those with disease, and assessment of the correlation between biomarker levels (or expression patterns of biomarkers) and clinical measurements that define disease status (15).

1.1 Biomarkers of Cardiovascular Disease

Biochemical markers play a crucial role in accurate diagnosis of CVD and, more importantly, in assessing risk and directing appropriate therapy that improves clinical outcome. Development and utilization of biomarkers has evolved substantially over the past three decades. The earliest biomarkers, such as alanine aminotransferase and lactate dehydrogenase, have fallen out in use with the development of more sensitive and specific assays for creatine kinase isoenzyme MB (CK-MB) and particularly cardiac troponin. Cardiac troponin T or I (cTnT o cTnI) measurements are now considered surrogates for necrosis and myocardial infarction (MI) when elevated in the setting of acute cardiac ischemia.

Biomarkers have provided important information for the clinical assessment of patients with suspected MI patients since the early 1950s. As displayed [Figure 2], utilization of biomarkers has evolved substantially over the past 30–40 yrs.

Biomarkers were previously considered to be one of the three important variables, along with changes on the electrocardiogram (ECG) and clinical signs and symptoms, necessary for the diagnosis of MI as defined by the World Health Organization (WHO) in 1979 (18*)*. The biomarkers cTnT and cTnI are now designated as surrogates for necrosis and MI when elevated in the setting of acute cardiac ischemia, according to the consensus document of the European Society of Cardiology (ESC) and the American College of Cardiology (ACC) (19).

The overall expectation of a CVD biomarker is to enhance the ability of the clinician to optimally manage the patient. For instance, in a person with chronic or atypical chest pain, a biomarker may be expected to facilitate the identification of patients with chest pain of an ischemic etiology (angina). In a patient presenting to the emergency room with acute severe chest pain (suspected acute coronary syndrome, ACS), a biomarker may help to differentiate patients with an acute MI from those with unstable angina (e.g., troponin I or T), acute pulmonary embolism (e.g., D-dimer or ventilation perfusion scan), or an AD (e.g., transesophageal echocardiogram) in a timely fashion to facilitate targeted management.

In addition to their diagnostic utility, the ability of cardiac biomarkers to facilitate risk assessment in chest pain patients has allowed emergency physicians and cardiologists to rapidly identify and treat higher-risk patients with suspected ACS.

However, the sensitivities of these cardiac biomarkers obtained on initial presentation may be poor and are dependent on the time from the onset of symptoms to presentation, the duration of ischemia, and the amount of myocardial tissue involved (20).

2. AORTIC DISSECTION

Aortic dissection (AD) is an acute CVD associated with high mortality and morbidity (21) and is the most frequent and serious form of acute aortic syndrome (AAS).

Knowledge regarding the incidence of AD in the general population is limited. Studies suggest an incidence of 2.6 to 3.5 cases per 100 000 person-years (22-24).

AAS is an acute process in the aortic wall involving a weakening of the medial layer with the risk of aortic rupture and other complications. It consists of three disease entities: aortic dissection, intramural hematoma, and penetrating atherosclerotic ulcer (25). It has an incidence of around 30 cases per million per year, of which 80% are aortic dissections, 15% intramural hematomas, and 5% penetrating atherosclerotic ulcers (26, 27). The ascending aorta is affected in 60% of cases (type A) and unaffected in 40% (type B). It mainly affects men (70%), with a mean age of 60 years as proved by the International Registry of Acute Aortic Dissection (IRAD) (28).

IRAD is an ongoing multi-national multi-centre registry started in 1996 that includes consecutive patients with acute AD at 22 aortic centers of 11 countries. The main objective of IRAD was to assess the etiology, mode of presentation, clinical features, management, and outcomes of patients with AD.

The dissection is characterized by separation of the layers within the aortic wall. Blood passes through the tear separating the intima from the media or adventitia, creating a false lumen. Propagation of the dissection can proceed in anterograde or retrograde fashion from the initial tear involving side branches and causing complications such as malperfusion syndromes, tamponade, or aortic valve insufficiency (29-32). Both acquired and genetic conditions share a common pathway leading to the breakdown in the integrity of the intima. The most common risk condition for AD is hypertension, with chronic exposure of the aorta to high pressures leading to intimal thickening, fibrosis, calcification, and extracellular fatty acid deposition (33, 34-36). Marfan's syndrome, vascular Ehlers-Danlos syndrome, annuloaortic ectasia, bicuspid aortic valve are genetic conditions that often cause acute AD. As displayed [Table 2], other risk conditions exist for AD (37).

The Stanford classification of AD distinguishes between type A and type B (38,39) [Figure 3]. In type A, the dissection involves the ascending aorta. In type B, only the descending aorta is involved. The DeBakey classification subdivides the dissection into 3 types, with type I dissection involving the entire aorta, type II dissection involving only the ascending aorta, and type III dissection sparing the ascending aorta and arch.

Acute AD of the ascending aorta is highly lethal, with a mortality of 1% to 2% per hour early after symptom onset (40,41).

Instantaneous onset of severe chest (85%) and/or back (46%) pain are characteristic presenting symptoms; however, abdominal pain (22%), syncope (13%), and stroke (6%) are common (28,42-44,45,46). Acute type A dissection is a surgical emergency. Medical management alone is associated with a mortality rate of nearly 20% by 24 hours after presentation, 30% by 48 hours, 40% at day 7, and 50% at 1 month. Even with surgical repair, mortality rates are 10% at 24 hours, 13% at 7 days, and nearly 20% at 30 days, as recently documented in the largest registry of AD, although randomized data are not available [Figure 4].

AD affecting the descending aorta is less lethal than type A dissection but not strikingly different regarding the clinical presentation. Instantaneous onset of severe back (64%) and/or chest (63%) pain are frequently reported symptoms, as is sudden abdominal pain (43%). Stroke is less common (21%), and presentation with an ischemic leg or peripheral ischemic neuropathy is encountered on occasion (1). Patients with uncomplicated type B dissection have a 30-day mortality rate of 10% [Figure 4]. Conversely, those who develop an ischemic leg, renal failure, visceral ischemia, or contained rupture often require urgent aortic repair; their mortality rate is 20% at day 2 and 25% at day 30 (44). Acute AD is a life-threatening medical emergency of the aorta; therefore, rapid diagnosis and timely management play an essential role in patient survival. The typical manifestation of acute AD is an acute onset of severe chest pain. However, symptoms may mimic more common disorders such as myocardial ischemia or stroke, and physical findings may be absent or suggestive of a diverse range of other conditions (47,48). The diagnosis of AD begins with clinical suspicion, which is the most crucial step in diagnosing this disease that should be confirmed rapidly and accurately, preferably with an easily available non-invasive modality.

The most frequently used modalities to identify AD are computerized tomography (CT), magnetic resonance imaging (MRI), transesophageal echocardiography (TEE), and angiography. In various studies, each of these imaging techniques has been reported to have high sensitivity, specificity and diagnostic accuracy.

A recently published metaanalysis (49) showed that diagnostic accuracy is practically the same (95%-100%) for CT, TEE, and MRI. Most patients require multiple imaging studies to diagnose and characterize AD.

The best combination for correctly diagnosing acute AD and its complications is CT and transthoracic echocardiography (TTE) (50, 51) [Table 3].

The choice of initial imaging modality may reflect availability rather than preference. Although TEE is accurate and can be performed quickly at the bedside with minimal risk, CT was the most common initial assessment performed [Table 4].

Despite recent reports of high sensitivity and specificity of MRI, it was rarely used as a first diagnostic imaging method (52, 53).

Availability, time delay, restricted ability to monitor patients during imaging, and incompatibility with implanted metal devices are likely explanations for its limited use. Aortography, previously the criterion standard, was used infrequently, and rarely as the initial study method. Despite improved diagnostic and therapeutic techniques, overall in hospital mortality for acute AD was 27.4% (25).

Because of the limitations of the various diagnostic modalities and the fact that many medical facilities do not have the equipment and expertise necessary to perform some or all of these test, many studies evaluated the role of biomarkers in diagnosis of AD. In suspected cases, diagnostic speed is of utmost importance. Bedside tests like the ECG, chest radiograph, and TTE cannot rule out the diagnosis of AD (41). More advanced imaging modalities can refute the diagnosis, but they are either semi-invasive (*i.e.*, TEE) or time-consuming and are not available in the emergency departement setting (*i.e.*, CT scanning, MRI, and aortography). Moreover, the accuracy of these tests is dependent on their performance and interpretation by skilled individuals. So, a simple and quick laboratory test to rule out AD would be of great value.

Until now, laboratory testing has played only a minor role in the assessment of acute AD, and tests are performed only for the exclusion of other diseases. Bedside specific biomarkers are not yet in clinical use, although biochemical diagnosis of AD may become feasible according to studies that have been done. In suspected AD, swift non invasive diagnostic imaging is advised to differentiate conditions requiring immediate action (involvement of the ascending aorta) from less dramatic scenarios (48, 53, 54).

2.1 Biomarkers of Aortic Dissection

Although newer diagnostic methods have greatly improved the diagnosis of AD, the diagnosis is still frequently missed today.

Because the clinical manifestations of AD are often diverse, the clinical presentation may mimic signs and symptoms of other diseases. A high level of suspicion maintained by the physician is therefore vital to definite diagnosis. The newer and preferred diagnostic methods (e.g., MRI, CT, TTE and TEE) are still limited by availability and technique. Also, the patients may be hemodynamically unstable, with diagnostic methods difficult to perform, and thus the diagnosis of the disease, even with available diagnostic equipment, may remain obscure (55).

A reliable biochemical diagnostic method for AD would be beneficial. Various biomarkers that can facilitate the diagnosis of AD have been studied in recent years [Table 5].

Research on the behavior of these and other new biomarkers may modify diagnostic strategies regarding AD in the near future and be of great assistance in carrying out the differential diagnosis between the diseases that are accompanied by chest pain.

Smooth muscle myosin heavy chain (SMMHC) was the initial pioneering discovery to showed that detection of circulating smooth muscle protein released from the aortic medial layer could be used to diagnose the disease (56-59).

AD is characterized by disruption of the aortic media by blood entering through a laceration of the luminal vascular wall. The insult causes extensive damage to smooth muscle cells of the media, leading to release of structural proteins of the smooth muscle cells, including SMMHC, into the circulation.

A rapid 30-minute immunoassay of SMMHC has been developed (58), showing high sensitivity and specificity, but the test is not widely used today. Patients with acute AD who presented within 3 hours after onset had elevated levels of circulating SMMHC protein. The temporal course of circulating SMMHC levels showed peak levels at onset with rapid normalization of level within the initial 24 hours [Figure 5]. In these patients, the assay had a sensitivity of 90.9%, within the first 12 hours after onset of AD, a specificity of 98% compared with healthy volunteers, and a specificity of 83% compared with patients who had acute MI (59).

The sensitivity and specificity of this assay in the first 3 hours after onset are similar if not superior to those of TTE (sensitivity, 59% to 85%; specificity, 63% to 96%), conventional CT (sensitivity, 83% to 94%; specificity, 87% to 100%), or aortography (sensitivity, 88%; specificity, 94%). However, the assay's sensitivity and specificity were lower than those of TEE (sensitivity, 98% to

99%; specificity, 77% to 97%), helical CT (both almost 100%), or MRI (both 98%) (52,53,61) [Figure 6].

It is important to note that because this assay is the first available biochemical diagnostic tool for AD, comparison with these established diagnostic methods (all of which are imaging procedures) provides only an estimation of its performance. Another important point is that biochemical testing can be done at a fraction of the cost of CT or MRI (approximately 10%) and is similar in cost to measuring cardiac enzymes (for example, myoglobin or troponin). The cost of a relatively inexpensive blood test is likely to outweigh the small risk for overlooking or failing to exclude the diagnosis of AD. In addition, manual or automated measurements can be performed easily in a similar manner to that of other conventional enzyme immunoassays.

The assay shows tremendous clinical possibilities, providing an easy, fast, and accurate method for screening of AD. The biochemical method shows promise in the differential diagnosis as well, as exemplified by acute MI. In the cardiovascular institution, as parameter of the clinical assessment, the method may play an assisting role in the diagnosis, along with other available diagnostic methods. In the facilities that do not have available diagnostic instruments (e.g., CT, TEE), the biochemical method may provide a highly useful tool for screening of AD in patients presenting with chest pain and to aid in the clinical judgment of the assessment and management of such cases.

To further develop additional markers, investigators have examined the role of creatine kinase BB-isozyme, which is selectively expressed in smooth muscle and is also elevated in this disease (60).

In particular, the BB-isozyme is preferentially expressed in smooth muscle and in brain in contrast to the MB-isozyme which is restricted to cardiac muscle and used in the diagnosis of acute MI and the MM-isozyme which is limited to skeletal muscle. A study has shown that creatine kinase BB-isozyme is elevated in patients with AD (61). The analysis showed that peak level for creatine kinase BB-isozyme may be delayed as compared to SMMHC which may allow for use of differential diagnostic temporal profiles similar to use of multiple cardiac marker in acute MI.

The combination of SMMHC and creatine kinase BB-isozyme showed promise for biochemical diagnosis of acute AD by circulating smooth muscle proteins [Figure 7].

Additional biochemical markers have been studied more into depth, including acute-phase reactants such as the white blood cell count, C-reactive protein, fibrinogen, and D-dimer.

D-dimer (DD) is a typical degradation product of cross-linked fibrin. Elevated DD levels generally can be seen with intravascular activation of the coagulation system and secondary fibrinolysis, in particular in patients with malignancies (62), disseminated intravascular coagulation (63), severe infections, complicated renal disease, recent trauma or surgery, and following fibrinolytic therapy (64).

Following an incidental observation, the relationship between elevated DD levels and acute AD has been systematically investigated (65).

The result of the DD test was positive in all patients with AD. The degree of the elevation was correlated to the delay from the onset of symptoms to laboratory testing and showed a trend to the extent of the dissection, but not to the outcome.

The DD test has demonstrated its usefulness in diagnosing AD, especially after the first 6 h (66). This suggest that testing for DD should be part of the initial assessment of patients with chest pain, especially if AD is suspected. A negative test result makes the presence of the disease unlikely.

More recently, soluble elastin fragments (sELAF) have been measured in the serum of patients with acute AD (67).

Elastin is one of the major structural matrix proteins of the arterial wall. Mature elastin is composed of soluble elastin subunits, which are intermolecularly cross-linked into a fibrous network (desmosine and isodesmosine formation) and thus construct a highly polymerized insoluble protein. The main pathological feature of the aortic media in acute AD is a higher grade of elastin degradation (68-71). Once an initial tear is formed, the dissection tends to expand to the degraded elastin layers, along with an inflammatory infiltrate, a major source of proteolytic enzymes such as elastases and metalloproteinases, which thus dramatically promote the fragmentation process of the elastin network in the media (70-72). As a result, sELAF are supposed to be released into the circulating blood. Therefore, sELAF in the serum might be a new and potentially useful variable for aid in the diagnosis of acute AD.

For this reason, it was developed an ELISA system for measuring sELAF in the serum that was reliable and reproducible. Using this system, it was demonstrated that acute AD patients within 48 hours after the onset showed an increase in the sELAF levels in serum.

In the last years, many studies have demonstrated that the elevation of matrix metalloproteinases (MMPs) might represent an opportunity to diagnostically characterize acute or chronic aortic processes not only in aterosclerotic aneurysms but also in AD (73).

AD is characterized by an acute phase of medial dissection and a subacute-chronic phase of vessel wall repair. MMPs, through degradation of extracellular matrix, may play an important role in these processes.

Recently, the potential diagnostic role of MMP-9 and MMP-2 in different phases of AD has been examined (74). MMP-9 plasma levels were increased in patients affected by type A and type B AD presenting within 1 h from onset of symptoms compared to controls. No differences were detected in MMP-2 plasma levels compared to controls. In type B AD, mean MMP-9 plasma levels increased significantly from hospital admission to 2-month follow-up. Conversely, no difference in

MMP-2 plasma levels was evident during follow-up. The expression of MMP-9 was evident at immunohistochemistry in the acute phase, whereas a marked expression was detected in the subacute phase. This pilot study suggests that the acute and subacute phase of both type A and type B aortic dissection is characterized by an increase of MMP-9 plasma levels. A marked increase is also evident in the subacute phase of medically treated type B AD as an expression of aortic wall remodelling. An increase of proteolytic activity could accompany attempts of the dissected aorta to heal itself but such a phenomena might further weaken the aortic wall, predisposing it to dilation and/or rupture.

The cardiac markers are an important parameter but are most potent when are used in combination with other diagnostic measures such as the imaging techniques, so each contributes to different diagnostic information. For AD, biochemical testing will likely show an optimal effect when is used in combination with imaging according to the ideal diagnostic algorithm [Figure 8].

3. AIM OF STUDY

The purpose of study is:

- to analyze the main demographic characteristics (sex, age) and a common symptom of CVD, as chest pain, in patients with suspected or confirmed AD and, thus, to assess the timeliness of a early biomarker for differential diagnosis;
- to enstablish the utility of the troponin-like protein of smooth muscle, calponin, as a potentially reliable biomarker for AD. In paticular, this study was conducted based on previous studies which showed that smooth muscle proteins released from the aortic medial smooth muscle cells at time of aortic insult can allow for biochemical detection of the disease as shown for the smooth muscle proteins, SMMHC and CK BB-isozyme.

Calponin was first isolated from chicken gizzard smooth muscle as a 34 kDa actin- and calmodulin-binding protein (75) and later shown to exist as multiple isoforms: h1- (α or basic), h2- (neutral) and acidic calponins; β-calponin is an alternatively spliced variant of h1-calponin and it has been detected at the protein level only in smooth muscle cells of the urogenital tract.

The domain organization of the three genetic isoforms of calponins is highly similar: all molecules contain an N-terminal calponin homology (CH) domain, followed by a short linker sequence connecting the high affinity binding site (ABS1), the adjacent triple CLIK repeats harboring the ABS2, and the C-terminal tail [Figure 9].

The tissue-specific *h*1 variant is involved in the regulation of the contraction/relaxation cycle in smooth muscle, probably by blocking the weak binding site for myosin on actin (76) and plays a key role in stabilizing the structural integrity of blood vessels (77). In adult vertebrates, basic calponin expression is restricted to differentiated smooth muscle cells where it is localized to the contractile and cytoskeletal actin filaments.

Basic calponin expression is down-regulated when vascular smooth muscle cells re-enter the cell cycle and proliferate, changing from a contractile to a synthetic phenotype (as occurs in response to vascular injury), and basic calponin is a useful marker of the contractile phenotype of smooth muscle cells. During embryonic development, this isoform is expressed in other tissues, including the heart, but disappears during late fetal development.

The observations that loss of the smooth-muscle-specific calponin variant resulted in increased fragility of the blood vessels accompanied by decreased cell adhesion and causing frequent leakage of the vessels significantly underscore the hypothesis that calponin's function as a structural component of the actin cytoskeleton is of considerable biological significance for smooth muscle function and regulation.

Neutral (h2-) and acidic isoforms of calponin are expressed in smooth muscle and non-muscle cells, but at much lower levels.

The biological function of *h*2 calponin is less clear but based on localization studies this variant has been implicated in the organization of the actin cytoskeleton (78-79). Acidic calponin is thought to control neurite outgrowth and branching (80), and neuronal regeneration (81). Although calponins display a high degree of sequence similarity within the N-terminal two thirds of the molecule, they differ significantly in their C-terminal regions.

The role of the N-terminal type 3 calponin homology (CH) domain in calponin is not well understood (82) and a number of different functions have been assigned to this region including the binding to phospholipids and extracellular regulated Ser/Thr kinases (ERK) involved in MAP kinase signaling pathways. In general, however, the CH domain is dispensable for actin binding in vitro and in vivo and probably plays a role in targeting calponin to the cell cortex or in recruiting additional calponin-binding partners.

The three genetic isoforms of calponin, *h*1, *h*2 and acidic, are distinguished mostly by their individual C-terminal tail sequences. Deletion of these sequences beyond the last homologous residue Cys273 increases actin filament association for all three isoforms, indicating a negative regulatory role for the unique tail regions (83).

One common biological property of all three calponin isoforms is binding to actin filaments; however, the subtle differences in biological functions, which can be predicted from the tissue-specific expression patterns of the individual isoforms, have not been determined.

4. METHODS

The clinical study "A novel biochemical method for the diagnosis of aortic dissection", has been carried out with Biosite Incorporated, a pharmaceutical firm from San Diego (USA) which, through combined expertise in diagnostic discovery and commercialization, it is able to identify potential markers of disease and proteins with high diagnostic utility, apply validated disease markers to advanced testing platforms.

ISBEM ScpA, an advanced research centre in the biomedical and healthcare field, set up a multidisciplinary group (biologists, clinicians, biothecnologist and sociologist) whose main activity was to organize, carry out and manage the clinical study according to Good Clinical Practice (GCP). GCP is an international ethical and scientific quality standard for designing, conducting, recording, and reporting trials that involve the participation of human subjects. Compliance with this standard provides public assurance that the rights, safety, and well being of trial subjects are protected, consistent with the principles that have their origin in the Declaration of Helsinki, and that the clinical trial data are credible.

It has been create a Italian network among the divisions of the cardiac surgery, cardiology, vascular surgery and emergency room; forty-six major centres, from all over Italy, were selected to take part in the study.

The requirements for selection of clinical centres were the following:

- the health areas had to be accessible and near to big aggregations in order to register the epidemiological phenomenon of AD of a given territory and to obtain homogeneous study populations with regard to environmental factors , life styles and culture;
- there had to be at least one doctor per each clinical centre with a proven technical-scientific background interested in the study protocol and oriented to scientific cooperation.

The study started after the institutional review board approval was obtained and start up site visits took place for a correct and detailed information on the protocol and on the specific operating procedures to investigators.

Only the patients, who had expressed their consent, were included in the study; in fact, the Informed Consent is fundamental in compliance with the regulations and laws in force in the field of clinical trials and with the rules of GCP, and in accordance with ethical requirements; thus, a form for the informed consent was drafted with a relevant informative file for the patient.

The patients have been enrolled into the study based on the following criteria:

• Age 18 and older;

• Patients confirmed as having an AD;

• Patients suspected of having acute or chronic AD who were referred for one or more of the following imaging tests:
    - Evaluation for AD based on computed tomography (CT) scan;
    - Evaluation for AD based on trans-esophageal echocardiogram (TEE);
    - Evaluation for AD based on aortography/angiography;
    - Evaluation for AD based on magnetic resonance imaging (MRI);

• Patients who presented within 24hrs of symptom onset.

Plasma specimens, treated with anticoagulant EDTA, have been taken during hospitalisation using standard blood draw procedures. Samples have been collected at the following time points:

• Presentation (at the time of enrollment)
• 3 hours after enrollment
• 6 hours after enrollment
• 12 hours after enrollment
• 24 hours after enrollment
• 48 hours after enrollment
• 72 hours after enrolment

As the stability of the analytes was not known, extra precautions were necessary to ensure the stability of the analyte in plasma and whole blood specimens.

The following protocol has been applied:

1. *Within 45 minutes of collecting the blood, the samples should be centrifuged at 2000xg for ten minutes at 4 ºC to separate the plasma from the cells.*
2. *Using a single sheet of 20 barcode labels, affix one barcode label to the collection tube and one barcode label to the appropriate area on the Case Report Form.*
3. *Label five 2 ml cryotubes with the barcode number that matches the same number on the collection tube and Case Report Form.*
4. *Aliquot the plasma into the 2 ml cryotubes that are labelled with the barcode label.*
5. *Store the labeled sample in a –20 ºC or colder freezer within one hour of collecting the blood. If samples are to be stored longer than 5 days, transfer the samples to a –80 ºC or colder freezer.*
6. *Transfer the samples to Biosite Inc. on dry ice.*

All patient samples and patient information forms have been collected, processed, and sent to Biosite Inc. used for measurements in the present study.

Clinical data forms were completed for each of the patients with parameters including demographics, history, physical findings, management, imaging studies, and outcomes, as developed by the IRAD [Figure 10], and have been entered in an electronic database specifically created. All forms have been reviewed for clinical face validity and analytical internal validity. Moreover, in February 2007**,** external validation was performed by an audit group, which was set up by Biosite Inc.

Finally, it has been developed an immunoassay against circulating calponins for an initial studies to address their role in biochemical diagnosis of AD.

Monoclonal antibodies against full length recombinant acidic calponin, peptide fragments of basic calponin (peptides included amino acids 274-281 and 289-297 of basic calponin), and full length recombinant neutral calponin were derived, and sandwich-type enzyme immunoassays were generated by Biosite Inc. according to standard procedures and protocols.

The normal range was 2.04 ng/ml for acidic calponin, 124.31 ng/ml for basic calponin, and 14.08 ng/ml for neutral calponin (84) [Table 6].

Note that neutral calponin was not further pursued after initial analysis demonstrated a lack of correlation with AD.

5. STATISTICAL ANALYSIS

Continuous variables were reported as mean and their standard deviations. Categorical variables were described using frequency tables.

Associations between categorical variables were examined by using Chi –square.

Statistical analysis was performed using the SAS statistical software (SAS Institute Inc, Cary, NC) versions 8.2 per Microsoft Windows.

6. RESULTS

6.1 Results of Patients Characteristics

Consenting patients with suspected AD who presented to participating Italian centres between April 2004 and June 2007 have been enrolled in the study.

Four hundred and twelve patients (62,8 ± 13,4 years) have been enrolled including 151 (36,7%) with AD (60,9 ± 13,4 years) and 261 (63,3%) with an initial suspicion of AD but a different final diagnoses (63,8 ± 12,7 years) [Tables 7-9].

The other main diagnoses were: aortic aneurysm 21 (5,1%), angina 67 (16,3%), acute MI 58 (14,1%), pulmonary embolism 4 (1,0%), panic disorder 22 (5,3%) and other 89 (21,6%) [Table 10].

71,1% of enrolled patients were males (61,7 ± 12,9 years), of whom 100 (66,2%) with AD (59,5 ± 13,6 years) and 193 (73,9%) without AD (62,8± 12,4 years); the females were 28,9% with higher mean age (65,4 ± 12,9 years), of whom 51 (33,8%) with AD (63,7 ± 12,7 years) and 68 (26,1%) without AD (66,7± 12,9years) [Tables 8-9]. Therefore, the number of males with AD was twice as many females, but the females had mainly AD.

AD type A was identified in 76.8% of patients (61,5 ± 13,8 years) that they were mostly males (64,7%) but, also in this case, the prevalence of disease was bigger in females (35,3%). 23,2% of patients presented AD type B (59,0 ± 11,6 years): the majority, 71,4%, were males (60,7 ± 11,4 years), while the females (28,6%) had a mean age more lowland (54,8 ± 11,7 years of age) [Tables 11-12-13].

The chest pain was the most common presenting symptom; of 412 patients enrolled 319 (77,4%) complained of chest pain, including 111 (34,8%) with AD and 208 (65,2%) with other final diagnoses. In particular, of the 151 patients with AD 111 (73,5%) had pain, whilst 40 (26,5%) didn't; of the 261 non AD cases 208 (79,7%) presented pain, whilst 53 (20,3%) didn't [Tables 14-15].

Of note, pain was described as severe in 50,5% of patients of whom 71 (34,1%) with AD and 137 (65,9%) with other pathologies [Tables 16-17].

Furthermore, 173 (54,2%) patients referred that the chest pain had abrupt onset: 77 (44,5 %) patients had AD, whilst 96 (55,5%) did not have AD; in particular, the pain was characterized by a abrupt onset in 77 (69,4%) of the 111 patients with AD [Tables 18-19].

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6.2 Results of preliminary study with calponin

Of all enrolled patients into the international study, by today, the plasma specimens of subgroup of 217 patients including 59 cases of acute AD and 158 cases with an initial suspicion of AD but a different final diagnosis have been analysed [Table 20].

Of the 59 AD cases (59 ± 14.5 years of age), 34 were males (58%). The non-AD cases (63 ± 14.8 years of age) included 116 males (73%). The other final diagnosis included MI (n=37), angina pectoris (n=34), pulmonary embolism (n=3), non-dissecting thoracic aortic aneurysm (n=17) or uncertain but not AD (n=67).

Acidic calponin showed a greater than two-fold increase for all dissections presenting within the first 6 hrs of symptom onset (4.10 ng/ml, n=16; normal reference, 2.04 ng/ml) which was particularly notable for type A (4.70 g/ml, n=14) as compared to type B patients (2.84 ng/ml, n=2). Type A patients in the 6-12 hr range also showed elevations (5.08 ng/ml, n=16) but not type B patients (2.43 ng/ml, n=4). Levels began to drop-off in the 12-24 hr range for type A (3.23 ng/ml, n=13) and were not significantly elevated in type B patients (2.64 ng/ml, n=9). Patients without AD did not show elevations at any of the examined time points (0-6 hr, 2.29 ng/ml, n=52; 6-12 hr, 2.65 ng/ml, n=34; 12-24 hr, 2.62 ng/ml, n=72) [Figure 11].

Basic calponin showed a more than three-fold increase at 377.56 ng/ml (normal reference, 123.31 ng/ml) for all dissections when sampled within the first 6 hrs of symptom onset (n=16) which was similar for type A (379.04 ng/ml, n=14) and type B patients (316.24 ng/ml, n=2). The 6-12 hr time-window showed similar, greater than three-fold, elevations in type A patients (348.79 ng/ml, n=16) but with a drop-off for type B patients (171.96 ng/ml). Levels in both type A and type B patients had fallen in the later 12-24 hr group (all patients, 169.24 ng/ml, n=22; type A patients, 172.05 ng/ml, n=13; type B patients, 171.96 ng/ml, n=9). Patients without AD did not show elevations at any of the examined time points (0-6 hr, 166.70 ng/ml, n=52; 6-12 hr, 179.41 ng/ml, n=34; 12-24 hr, 159.98 ng/ml, n=72) [Figure 12].

Neutral calponin did not show elevations in any AD patient regardless of type or time from onset of symptoms (0-6 hr, 5.11 ng/ml, n=16; 6-12 hr, 18.17 ng/ml, n=21; 12-24 hr, 13.19 ng/ml, n=22; normal reference, 14.08 ng/ml). As expected, neutral calponin did not show elevations in non-AD controls (0-6 hr, 15.03 ng/ml, n=52; 6-12 hr, 8.19 ng/ml, n=34; 12-24 hr, 12.30 ng/ml, n=72).

Thus, acidic and basic calponins showed greater than two-fold and three-fold elevations respectively during the initial 6 hrs with type A and remained elevated through to 12 hrs. For type B dissection, acidic and basic calponin levels were elevated in the very early presenters (0-6 hrs)

but not afterward. Neutral calponin did not show disease-associated changes and was not further pursued.

Further analysis according to final diagnosis was done for acidic and basic calponin [Figure 13].

Sensitivity and specificity of detection of acute AD were also analysed by receiver operating characteristics (ROC) curves.

The optimal clinical decision limit value was determined from these ROC curve analyses which showed that the optimal value for acidic calponin was 2.8 ng/ml which resulted in a sensitivity of 50% and specificity of 87% for the initial 6 hrs, and 2.3 ng/ml which resulted in a sensitivity of 58% and specificity of 72% for the initial 24 hr period.

Similarly, the optimal value for basic calponin was 159 ng/ml which resulted in a sensitivity of 63% and specificity of 73% for the initial 6 hrs, and 139 ng/ml which resulted in a sensitivity of 50% and specificity of 66% for the initial 24 hr period. According to type, type A showed an optimal value for acidic calponin at 2.8 ng/ml which resulted in a sensitivity of 50% and specificity of 87% for the initial 6 hrs, and 2.3 ng/ml which resulted in a sensitivity of 58% and specificity of 72% for the initial 24 hr period. Similarly, the optimal value for basic calponin was 159 ng/ml which resulted in a sensitivity of 64% and specificity of 73% for the initial 6 hrs, and 141 ng/ml which resulted in a sensitivity of 50% and specificity of 67% for the initial 24 hr period.

The predictive values (negative and positive) as calculated with a prevalence of 1 in 10,000 were 0.84 and 0.56 in the initial 6 hrs and 0.84 and 0.41 in the initial 24 hrs, respectively, for acidic calponin, and 0.86 and 0.44 in the initial 6 hrs and 0.80 and 0.33 in the initial 24 hrs, respectively, for basic calponin. [Table 21] Importantly, both acidic and basic calponin had higher negative predictive values.

7. DISCUSSION

Although AD is a rare pathology, the creation of a big national network among the divisions of cardiac surgery, cardiology, vascular surgery and emergency room has been very useful as, not only has it enabled to enroll a significant number of patients with suspected AD, but it could also represent a valid tool to guarantee the uniform treatment of all patients suffering of this pathology.

From the descriptive analysis of data it is inferred that the majority of enrolled patients presented chest pain as the main symptom, and half of patients reported a severe pain with a abrupt onset.

The chest pain characterizes the onset of various and significant CVD, thus it is evident how important it is to have/do a timely differential diagnosis for those pathologies with a similar symptomatology, in many cases necessary for the patient's own survival; in particular, the majority of patients with AD showed chest pain thus the availability of a reliable biochemical test would have been useful, in association with imaging test, for an early diagnosis which is a prerequisite for improved treatment and survival.

There currently is no readily available, reliable, bedside biomarker assay for AD. Thus, this experience is promising for identification of a future serum biomarker or panel of markers that may aid in the more rapid diagnosis of AD.

The immunoassay developed against basic calponin showed the greatest elevations. Acidic calponin also showed diagnostic elevations, but disease-associated changes in neutral calponin levels was non-diagnostic. Analysis by type and time after onset showed that acidic calponin reliably detects AD within the first 12 hrs with superior performance in type A patients. Basic calponin showed superior performance for the first 6 hrs.

Importantly, calponin measurements allowed for detection of the disease in patients with a more delayed presentation (out to 12 hrs) which should be a welcome addition for diagnostic use in comparison with SMMHC which previously has been shown to possess superior accuracy for patients presenting within six hours after onset.

Combined use of calponin and SMMHC assays might allow for improved detection of acute AD by biochemical means as well as to potentially determine the time of onset.

This would be analogous to use of multiple diagnostic biomarkers for diagnosis of acute MI with the initial peak in myoglobin being followed by later elevations in CK MB-isozyme and troponins.

One of the strengths of the present study was that diagnostic performance of the assays was determined in patients who were enrolled on the basis of a clinical suspicion of AD and not by comparison with healthy controls. Thus, the test's accuracy reflects 'real-world' conditions.

Further, although a large number of patients of this rather uncommon disease has been examined, the breakdown analysis according to type and time after onset resulted in subgroups which were

limited in number and therefore did not allow for unequivocal results in certain cohorts (e.g. high AUC values in type B dissection for basic calponin based on two diseased cases).

8. CONCLUSIONS

The increasing ageing of the western population and the consequent increase of the aortic diseases make it more necessity to develop sensitive and specific instruments for a differential diagnosis.

The use of an efficient and rapid biochemical method, which can address toward specific diagnostic examination and relevant therapeutic choices in the shortest possible time, represents for the patient the fundamental “life-saving” selection.

The descriptive analysis of data shows that chest pain was the most common symptom in cases of AD but, given the relative frequency of chest pain in patients presenting to emergency department and the relative infrequency of AD, the availability of a reliable biochemical method could be of great assistance in carrying out the differential diagnosis between the diseases that are accompanied by chest pain.

Patients who present with abrupt onset of severe chest pain are straightforward candidates for imaging; a biochemical test to screen for dissection offers great potential to improve case finding and at the same time potentially reduce the expense of unnecessary imaging for patients who don't have AD.

Acid and basic calponins have the potential for use as an early diagnostic biomarker for AD but the results of this preliminary experience using an initial assay show moderate sensitivities and specificities with negative predictive values which should be further improved upon.

Future studies will be aimed at further improving the assay and determining the optimal combination of this and other biomarkers for diagnosis of AD

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TABLES

Table 1. Measures of biomarker test performance.
Adapted from reference 3

Sensitivity	is defined as the ability of a test to detect disease (condition of interest) when it is truly present, i.e., it is the probability of a positive test result given that the patient has the disease.
Specificity	is the ability of a test to exclude the disease (condition of interest) in patients who do not have disease, i.e., it is the probability of a negative test result given that the patient does not have the disease.
Predictive value	tells us how good the test is at predicting the true positives or true negatives, i.e., the probability that the test will give the correct diagnosis.
Positive Predictive Value	is the probability that a patient has the disease given that the test results are positive.
Negative Predictive Value	is the probability that a patient does not have the disease or condition given that the test results are indeed negative.
ROC curve	is a plot of the sensitivity versus specificity of a diagnostic test, in which the different points on the curve correspond to different cut points used to determine whether the test results are positive.
Prevalence	is defined as the prior probability of the disease before the test is performed.

Table 2. Risk Conditions for Aortic Dissection

Long-standing arterial hypertension

● Smoking, dyslipidemia, cocaine/crack

Connective tissue disorders

● Hereditary vascular disease
● Marfan syndrome
● Vascular Ehlers-Danlos syndrome (type 4)
● Bicuspid aortic valve
● Coarctation of the aorta
● Hereditary thoracic aortic aneurysm/dissection

Vascular inflammation

● Giant cell arteritis
● Takayasu arteritis
● Behcet's disease
● Syphilis
● Ormond's disease

Deceleration trauma

● Car accident
● Fall from height

Iatrogenic factors

● Catheter/instrument intervention
● Valvular/aortic surgery
● Side or cross-clamping/aortotomy
● Graft anastomosis
● Patch aortoplasty
● Aortic wall fragility

Table 3. Usefulness of imaging techniques in the diagnosis of acute aortic syndrome*(50)

	ANGIO	*TTE*	*TEE*	*CT*	*MRI*
Diagnostic accuracy	++	+	+++	+++	+++
Extension	+++	+	++	+++	+++
Entry port	++	+	+++	++	++
Aortic regurgitation	+++	+++	+++	-	+++
Effusion/tamponade	-	+++	+++	++	++
Arterial trunks	+++	+	+	+++	++
Periaortic bleeding	-	-	+	+++	+++

*Angio indicates catheter angiography; TEE, transesophageal echocardiography; TTE, transthoracic echocardiography; MRI, magnetic resonance imaging; CT, computerized tomography. +++ Excellent; ++ Good; + Sufficient; - Insufficient

Table 4. Advantages of imaging during the acute phase study (50)

	ANGIO	*TTE*	*TEE*	*CT*	*MRI*
Speed	+	+++	+++	++	+
Portability	-	+++	+++	-	-
Monitoring	+++	+++	+++	+	-
Availability	+	+++	++	+++	+
Tolerance	+	+++	++	+++	+

*Angio indicates catheter angiography; TEE, transesophageal echocardiography; TTE, transthoracic echocardiography; MRI, magnetic resonance imaging; CT, computerized tomography. +++ Excellent; ++ Good; + Sufficient; - Insufficient

Table 5. Biomarkers for diagnosis of AD.

Endothelial Marker
(von Willebrand Factor, Thrombomodulin, etc.)
Markers of the Smooth Muscle
(Smooth Muscle Myosin Heavy Chain, Creatine kinase, etc.)
Markers of the Adventitia/Extracellular Matrix
(Collagen, Elastin, Matrix Metalloproteinases, etc.)
Coagulation Markers
(D-dimer, etc.)
Inflammation Marker
(C- Reactive Protein, etc.)

Table 6. Normal reference range for assay (76).

	n	Average	Median	SD	95th percentile	99th percentile
Acidic calponin (ng/ml)	218	1.19	1.01	0.92	2.04	5.73
Basic calponin (ng/ml)	282	44.09	31.74	41.81	124.31	162.15
Neutral calponin (ng/ml)	230	12.61	5.00	64.72	14.08	52.65

Table 7: Enrolled patients according to diagnosis and gender. Period of enrollment April 2004- June 2007. Percentage of column.

Patients	Male		Female		Male and female	
	AV	%	AV	%	AV	%
AD	100	34,1	51	42,9	151	36,7
out AD	193	65,9	68	57,1	261	63,3
l	293	100,0	119	100,0	412	100,0

ource: Clinical Study "A novel biochemical method for the diagnosis of aortic dissection".
AD = Aortic Dissection; AV = Absolute Value.
p = 0,0868

Table 8: Enrolled patients according to diagnosis and gender. Period of enrollment April 2004- June 2007. Percentage of row.

tients	Male		Female		Male and female	
	AV	%	AV	%	AV	%
AD	100	66,2	51	33,8	151	100,0
out AD	193	73,9	68	26,1	261	100,0
l	293	71,1	119	28,9	412	100,0

ource: Clinical Study "A novel biochemical method for the diagnosis of aortic dissection".
AD = Aortic Dissection; AV = Absolute Value.
p = 0,0868

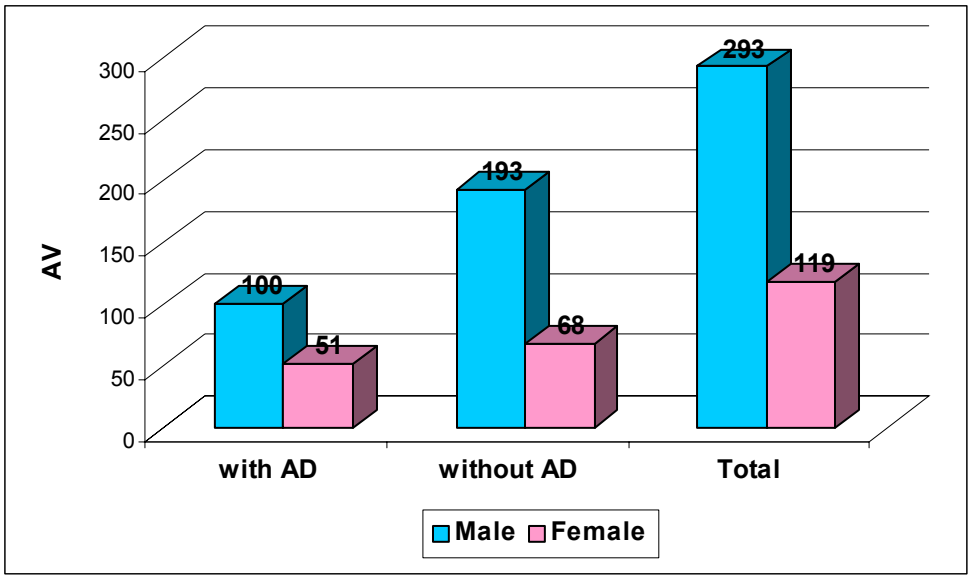


Table 9: Mean age and standard deviations of enrolled patients. Period of enrollment April 2004- June 2007.

Patients	Media	SD	Min	25%	Mediana	75%	Max
				Male			
with AD	59.50	13.70	18.63	52.29	59.85	69.11	87.00
without AD	63.98	12.96	33.59	54.15	64.73	74.30	88.54
				Female			
with AD	63.70	12.79	26.93	54.63	66.57	72.25	84.33
without AD	62.84	11.47	35.58	54.65	63.14	72.57	85.10
				Male and female			
with AD	60.92	13.51	18.63	52.68	62.24	70.03	87.00
without AD	63.69	12.59	33.59	54.54	64.62	72.83	88.59

Source: Clinical Study "A novel biochemical method for the diagnosis of aortic dissection".

D = Aortic Dissection; SD = Standard Deviations; Min = the minimun value; 25% = 25th percentile; =75th percentile; Max = the maximum value.

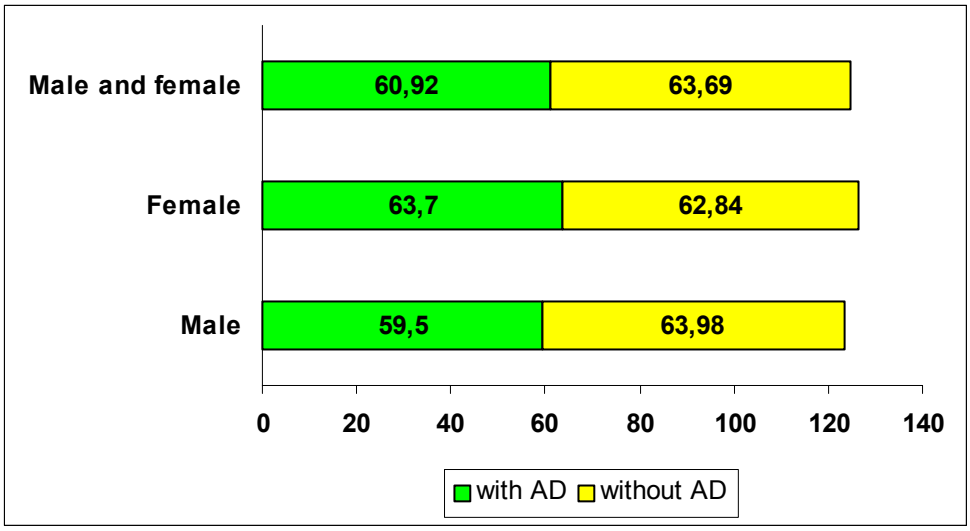


Table 10: Different diagnosis of enrolled patients. Period of enrollment April 2004- June 2007.

Diagnosis	AV	%
Type A AD	116	28,1
Type B AD	35	8,5
Aortic Aneurysm	21	5,1
Angina	67	16,3
Acute Myocardial Infarction	58	14,1
Pulmonary Embolism	4	1,0
Panic disorder	22	5,3
Other	89	21,6
Total	412	100,0

Source: Clinical Study "A novel biochemical method for the diagnosis of aortic dissection".
AD = Aortic Dissection; AV = Absolute Value.

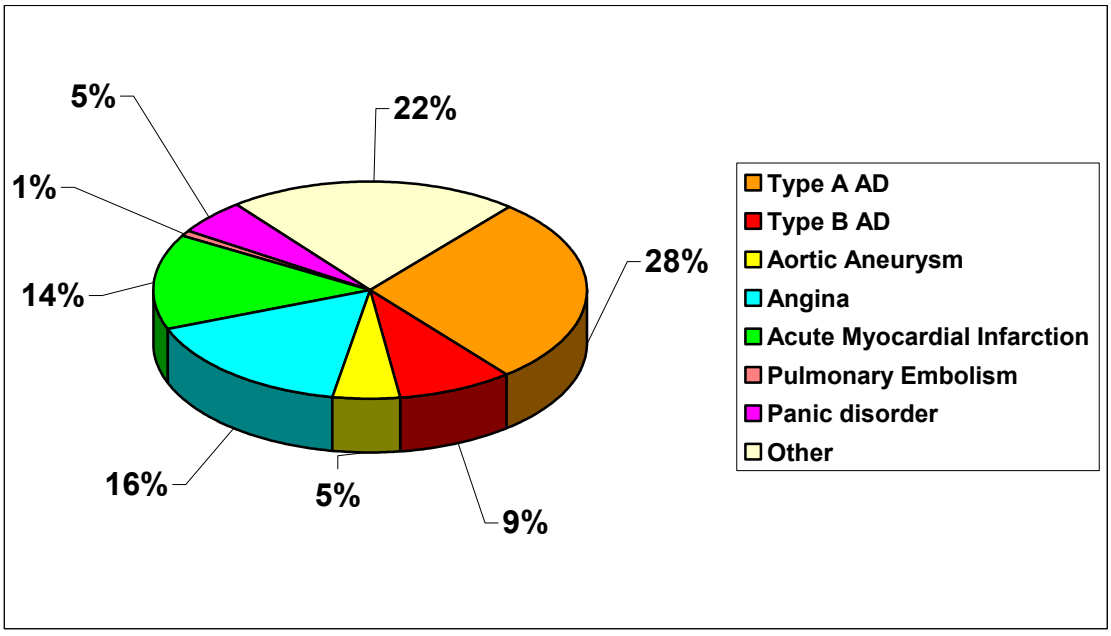


Table 11: Enrolled patients according to diagnosis of AD and gender. Period of enrollment April 2004- June 2007. Percentage of column.

tients	Male		Female		Male and female	
	AV	%	AV	%	AV	%
pe A AD	75	75,0	41	80,4	116	76,8
pe B AD	25	25,0	10	19,6	35	23,2
al	100	100,0	51	100,0	151	100,0

ource: Clinical Study "A novel biochemical method for the diagnosis of aortic dissection".
AD = Aortic Dissection; AV = Absolute Value.
p =0,4577

Table 12: Enrolled patients according to diagnosis of AD and gender. Period of enrollment April 2004- June 2007. Percentage of row.

Patients	Male		Female		Male and female	
	AV	%	AV	%	AV	%
Type A AD	75	64,7	41	35,3	116	100,0
Type B AD	25	71,4	10	28,6	35	100,0
Total	100	66,2	51	33,8	151	100,0

Source: Clinical Study "A novel biochemical method for the diagnosis of aortic dissection".
AD = Aortic Dissection; AV = Absolute Value.
p =0,4577

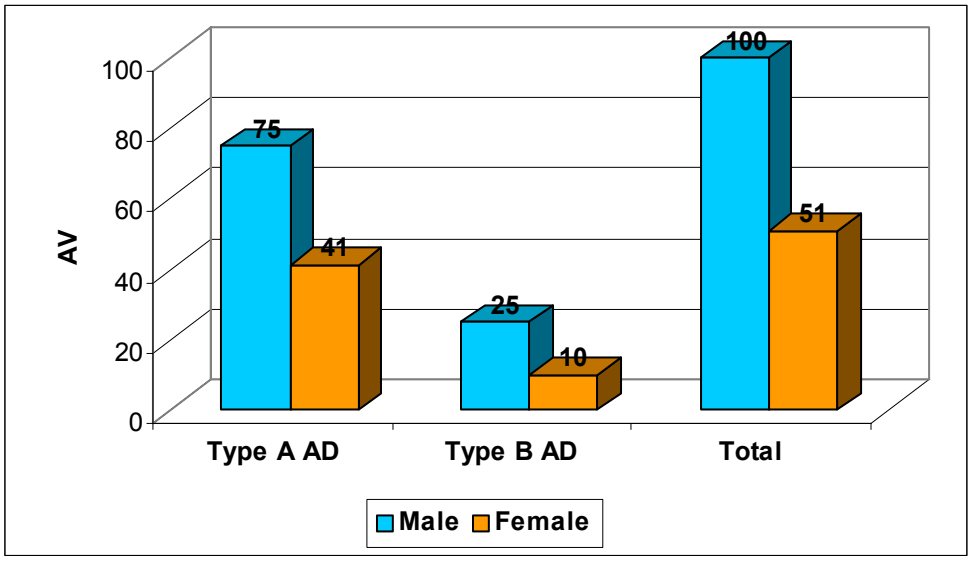


Table 13: Mean age and standard deviations of enrolled patients with AD. Period of enrollment April 2004- June 2007.

Patients	Media	SD	Min	25%	Mediana	75%	Max
				Male			
Type A AD	59.10	14.38	18.63	51.06	59.83	68.75	87.00
Type B AD	60.72	11.63	27.99	55.31	60.73	69.76	80.43
				Female			
Type A AD	65.88	12.07	26.93	60.49	68.97	74.96	84.33
Type B AD	54.78	12.30	41.36	43.03	53.38	64.96	75.16
				Male and female			
Type A AD	61.49	13.94	18.63	52.78	63.50	71.79	87.00
Type B AD	50.02	11.96	27.99	49.50	59.39	68.21	80.43

urce: Clinical Study "A novel biochemical method for the diagnosis of aortic dissection".

AD = Aortic Dissection; SD = Standard Deviations; Min = the minimun value; 25% = 25th percentile; 75% =75th percentile; Max = the maximum value.

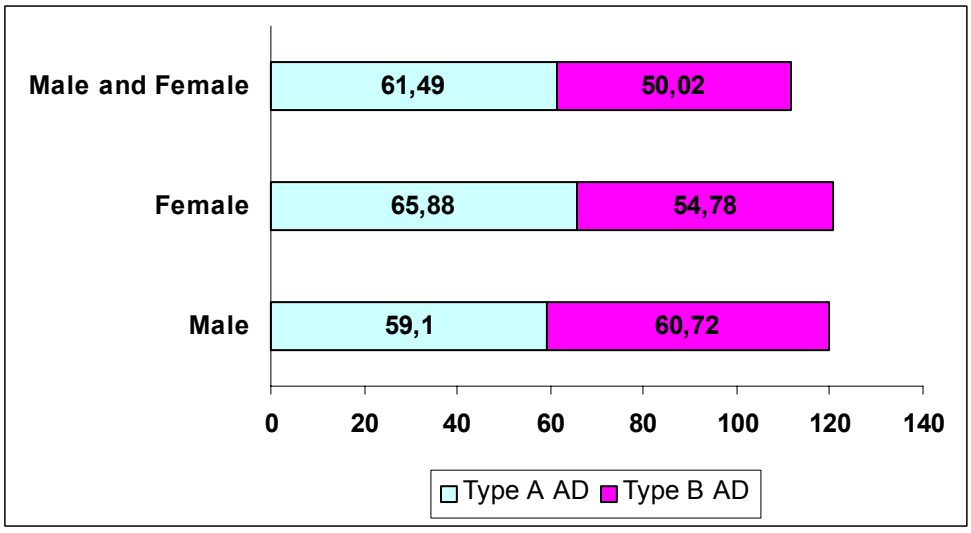


Table 14: Enrolled patients with chest pain. Period of enrollment April 2004- June 2007. Percentage of column.

tients	with chest pain		without chest pain		Total	
	AV	%	AV	%	AV	%
D	111	34,8	40	43,0	151	36,7
thout AD	208	65,2	53	57,0	261	63,3
l	319	100,0	96	100,0	412	100,0

ource: Clinical Study "A novel biochemical method for the diagnosis of aortic dissection".

D = Aortic Dissection; AV = Absolute Value.

p 0.1480

Table 15: Enrolled patients with chest pain. Period of enrollment April 2004- June 2007. Percentage of row.

tients	with chest pain		without chest pain		Total	
	AV	%	AV	%	AV	%
D	111	73,5	40	26,5	151	100,0
thout AD	208	79,7	53	20,3	261	100,0
l	319	77,4	93	22,6	412	100,0

ource: Clinical Study "A novel biochemical method for the diagnosis of aortic dissection".

AD = Aortic Dissection; AV = Absolute Value.

p 0.1480

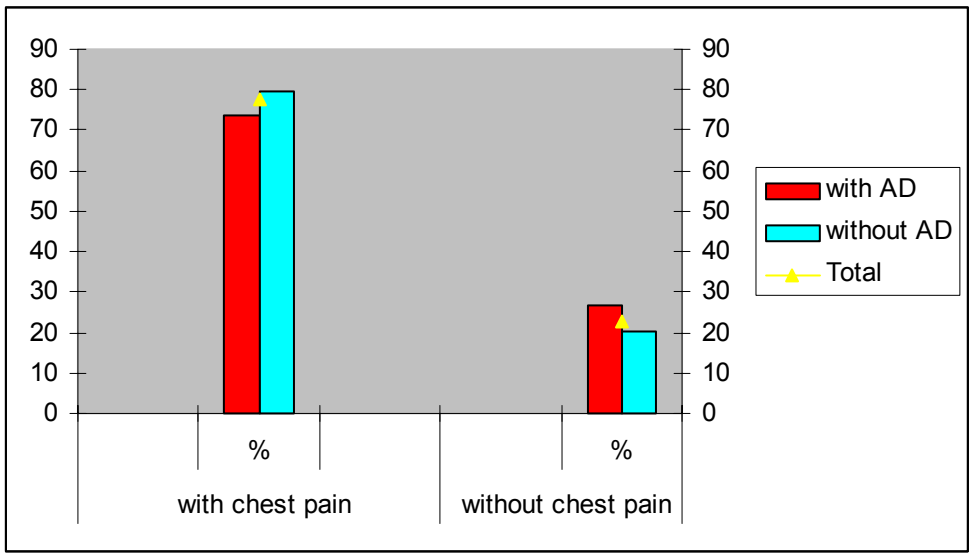


Table 16: Pain severity of patients. Period of enrollment April 2004- June 2007. Percentage of column.

Patients	Mild		Severe		Worst ever		Other		Total	
	AV	%	AV	%	AV	%	AV	%	AV	%
with AD	15	21,4	71	34,1	23	79,3	42	40,0	151	36,7
without AD	55	78,6	137	65,9	6	20,7	63	60,0	261	63,3
Total	70	100,0	208	100,0	29	100,0	105	100,0	412	100,0

Source: Clinical Study "A novel biochemical method for the diagnosis of aortic dissection".

AD = Aortic Dissection; AV = Absolute Value

p <.0001

Table 17: Enrolled patients with severe chest pain. Period of enrollment April 2004- June 2007. Percentage of row.

Patients	Mild		Severe		Worst ever		Other		Total	
	AV	%	AV	%	AV	%	AV	%	AV	%
with AD	15	10,0	71	47,0	23	15,2	42	27,8	151	100,0
without AD	55	21,1	137	52,5	6	2,3	63	24,1	261	100,0
Total	70	17,0	208	50,5	29	7,0	105	25,5	412	100,0

Source: Clinical Study "A novel biochemical method for the diagnosis of aortic dissection".

AD = Aortic Dissection; AV = Absolute Value

p <.0001

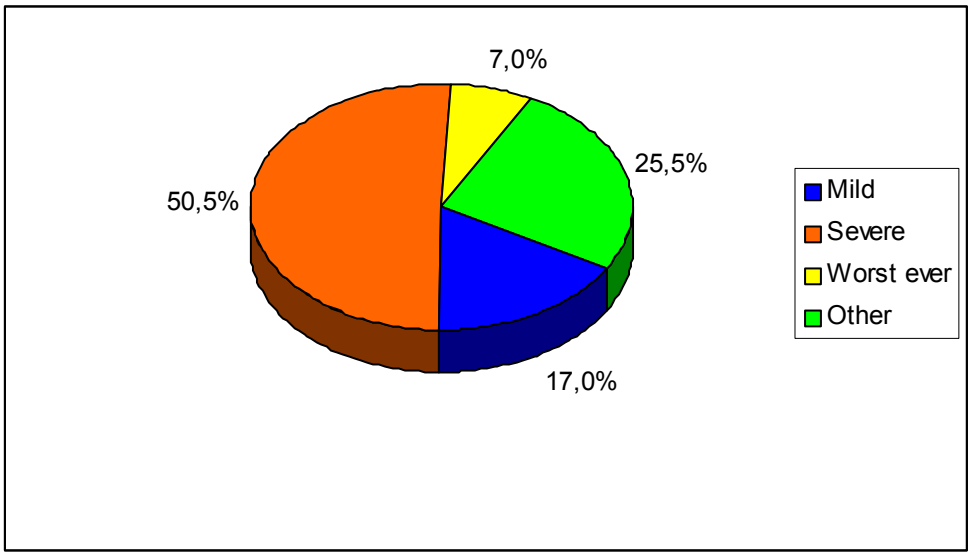


Table 18: Enrolled patients with a abrupt onset of chest pain. Period of enrollment April 2004- June 2007. Percentage of column.

tients	with abrupt onset		without abrupt onset		Total	
	AV	%	AV	%	AV	%
h AD	77	44,5	34	23,3	111	34,8
hout AD	96	55,5	112	76,6	208	65,2
tal	173	100,0	146	100,0	319	100,0

ource: Clinical Study "A novel biochemical method for the diagnosis of aortic dissection".
= Aortic Dissection; AV = Absolute Value.
01

Table 19: Enrolled patients with a abrupt onset of chest pain. Periodof enrollment April 2004- June 2007. Percentage of row.

tients	with abrupt onset		without abrupt onset		Total	
	AV	%	AV	%	AV	%
h AD	77	69,4	34	30,6	111	100,0
hout AD	96	46,2	112	53,8	208	100,0
tal	173	54,2	146	45,8	319	100,0

ource: Clinical Study "A novel biochemical method for the diagnosis of aortic dissection".
AD = Aortic Dissection; AV = Absolute Value.
01

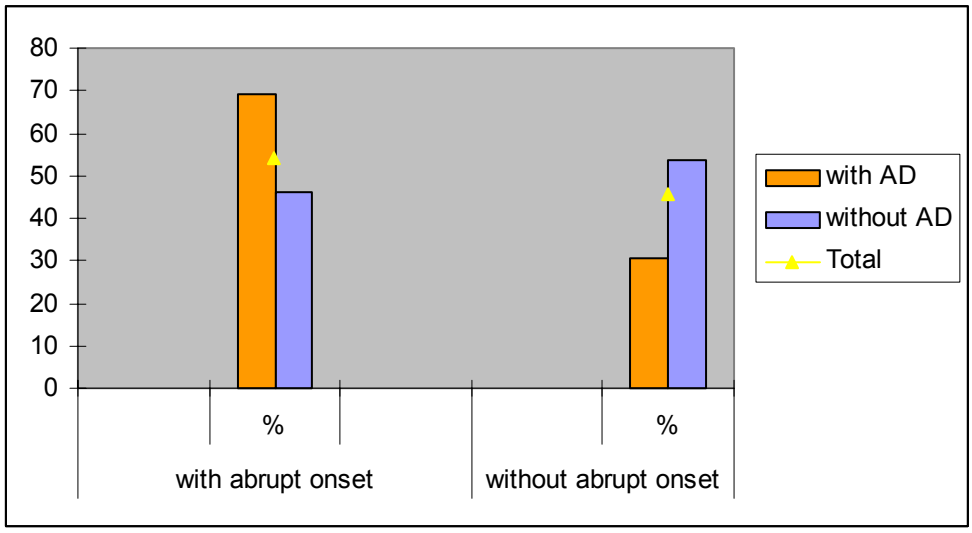


Table 20. Patient demographics of the study population

Acute aortic dissection	59 cases (34 males, 59 ± 14.5 years)
Dissection type (Stanford classification)	
Type A	43 cases
Type B	15 cases
Type undeterminated in one case	
Presentation time from onset	
< 6 h	16 cases (type A 14 cases, type B 2 cases)
6- 12 h	20 cases (type A 16 cases, type B 4 cases)
12-24 h	22 cases (type A 13 cases, type B 9 cases)
Non-aortic dissection	158 cases (116 males, 63 ± 14.8 years)
Final diagnosis	
Myocardial infarction	37 cases
Angina pectoris	34 cases
Pulmmonary embolism	3 cases
Thoracic aortic aneurysm	17 cases
Uncertain but not aortic dissection	67 cases
Presentation time from onset	
< 6 h	52 cases
6- 12 h	34 cases
12-24 h	72 cases

Table 21. Diagnostic performance of assay.

	AD				Type A				Type B			
	Number of patients	Number of non-patients	ROC AUC	P-value	Number of patients	Number of non-patients	ROC AUC	P-value	Number of patients	Number of non-patients	ROC AUC	P-value
Early samples: AD (0-6 h) vs. non-AD (0-6 h)												
Acidic calponin, a	16	52	0.63	0.11	14	52	0.66	0.07	2	52	0.46	0.85
Basic calponin, b	16	52	0.67	0.04	14	52	0.65	0.10	2	52	0.82	0.13
Neutral calponin	16	52	0.42	0.33	14	52	0.39	0.20	2	52	0.64	0.49
All samples AD: (0-24 h) vs. non-AD (0-24 h)												
Acidic calponin, c	59	158	0.63	0.00	43	158	0.65	0.00	15	158	0.54	0.57
Basic calponin, d	59	158	0.58	0.08	43	158	0.59	0.08	15	158	0.54	0.63
Neutral calponin	59	158	0.50	0.99	43	158	0.49	0.77	15	158	0.56	0.48

NPV,negative predictive value; PPV, positive predective value.

a, NPV: 0.84; PPV: 0.56.

b, NPV: 0.86; PPV: 0.44.

c, NPV: 0.84; PPV: 0.41.

d, NPV: 0.80; PPV: 0.33.

FIGURES

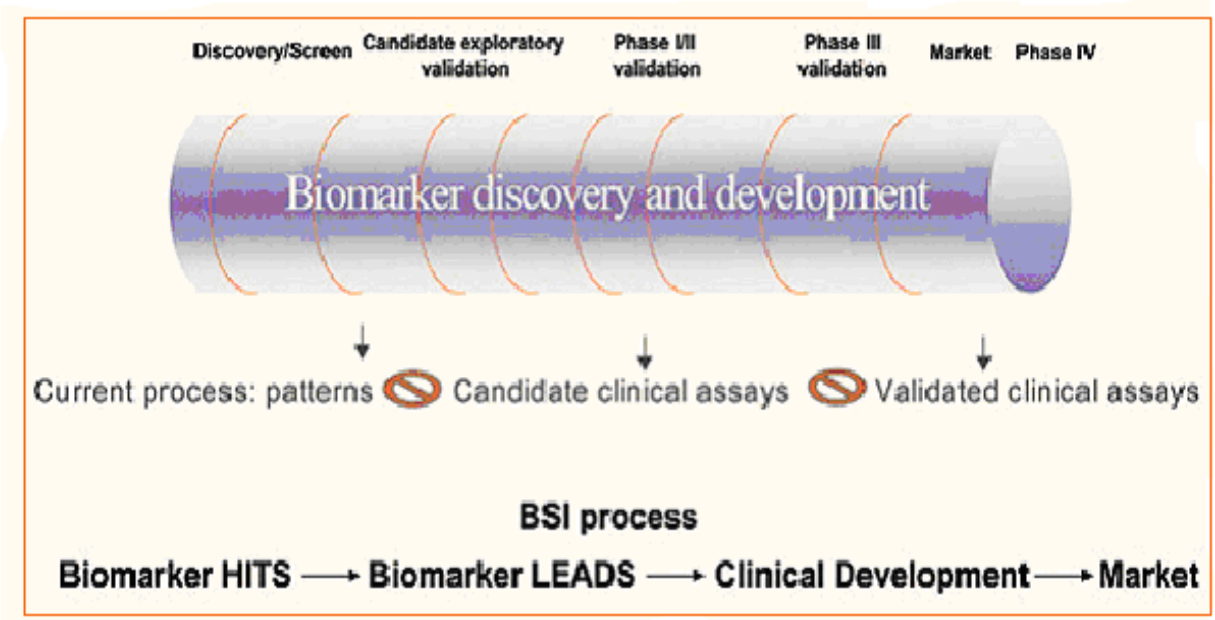


Figure 1. The development of biomarker: from discovery to delivery/publication/release.

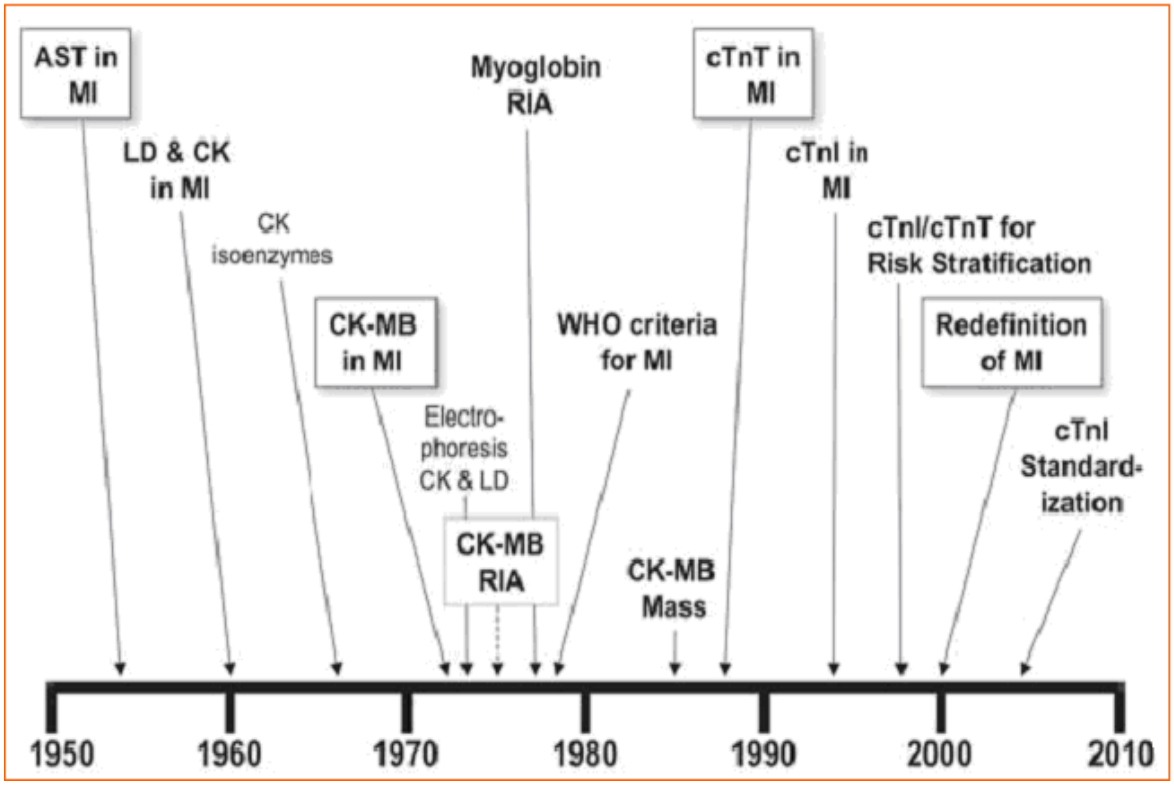


Figure 2. Evolution of cardiac biomarkers. MI, myocardial infarction; AST, aspartate transaminase; LD, lactate dehydrogenase; CK, creatine kinase; RIA, radioimmunoassay

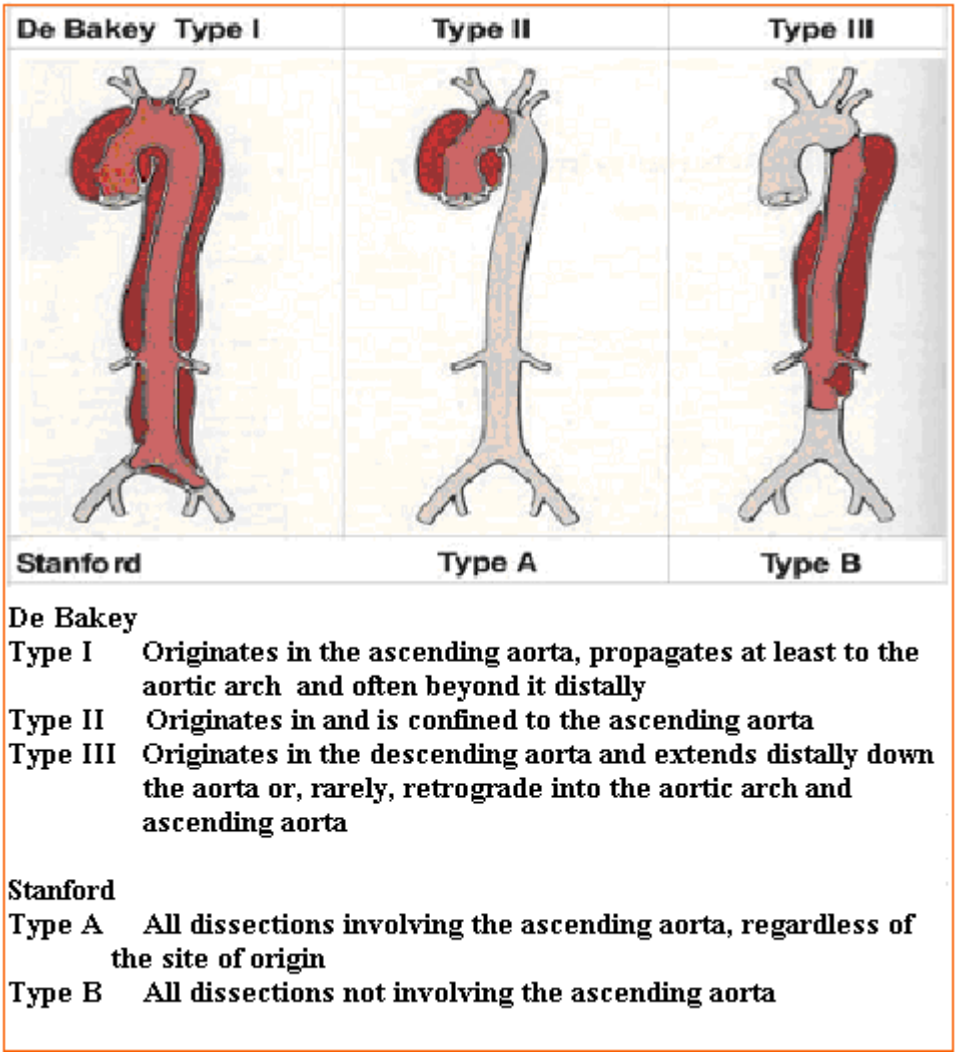


Figure 3. The most common classification systems of thoracic aortic dissection: Stanford and DeBakey.

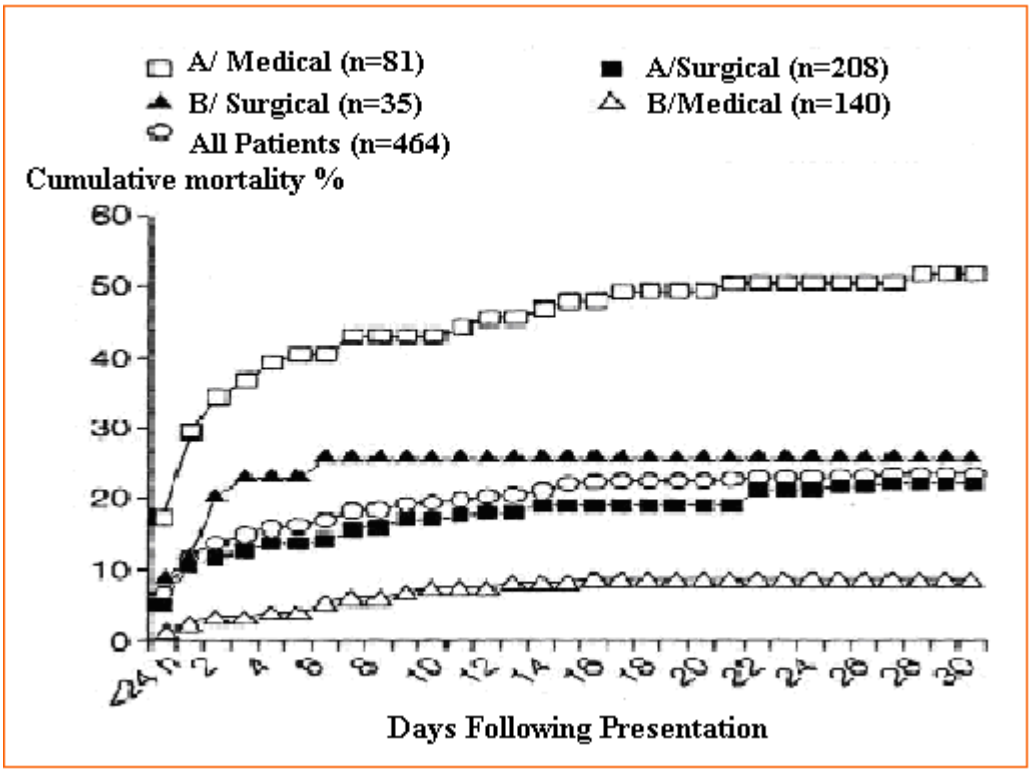


Figure 4. Thirty-day mortality in 464 patients from the IRAD registry stratified by medical and surgical treatment in both type A and type B aortic dissection. Adapted from reference 3.

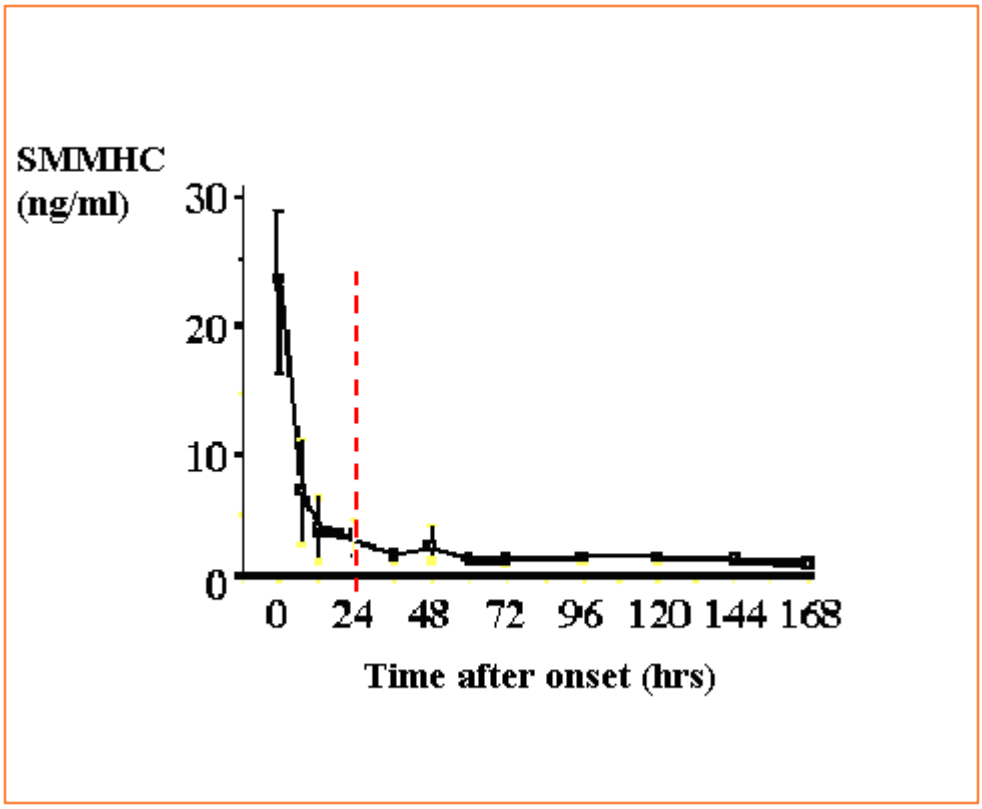


Figure 5. Time course of serum smooth muscle myosin heavy chain levels in patients with aortic dissection Note that peak levels are at onset. Rapid reductions in levels are found during the first 24 hours (58).

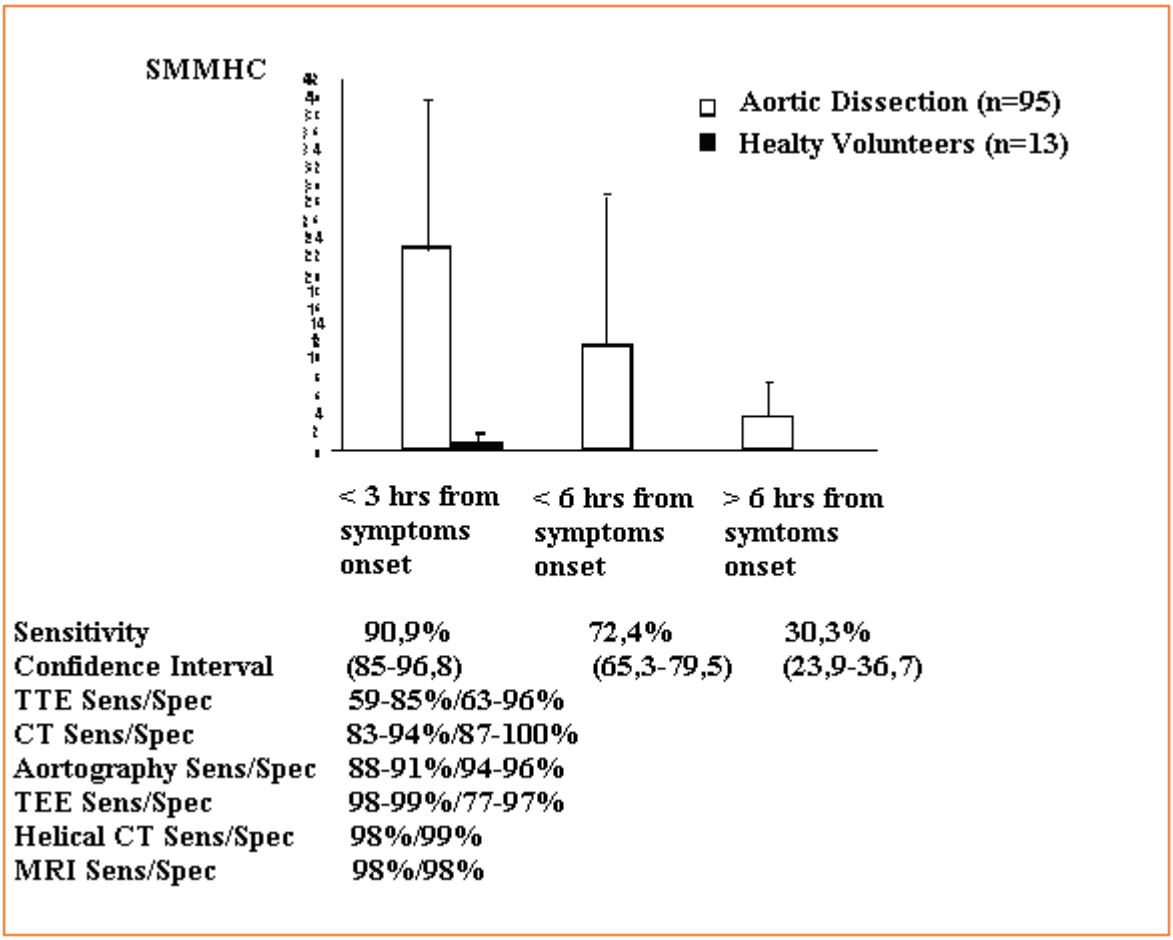


Figure 6. Temporal sensitivity curves based on the site of the entry tear for smooth muscle myosin heavy-chain protein measurement with a cutoff level of 2.5 μg/L (59).

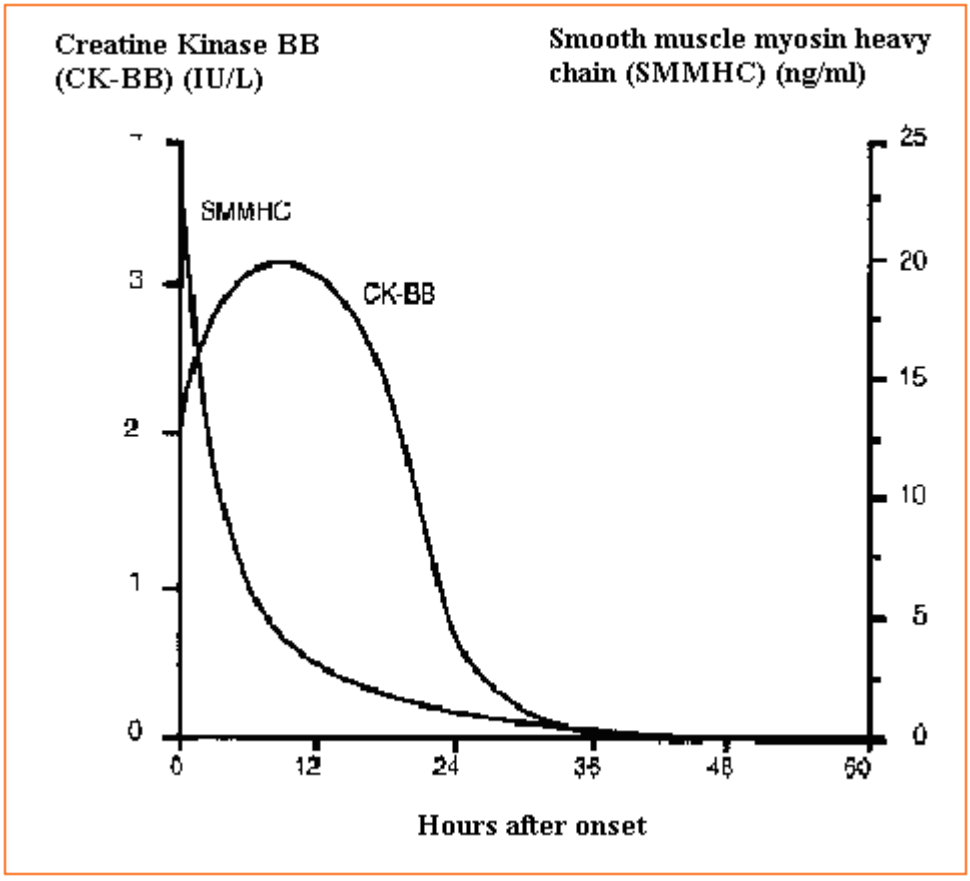


Figure 7. Temporal profiles of smooth muscle myosin heavy chain and creatine kinase BB-isozyme in AD (61).

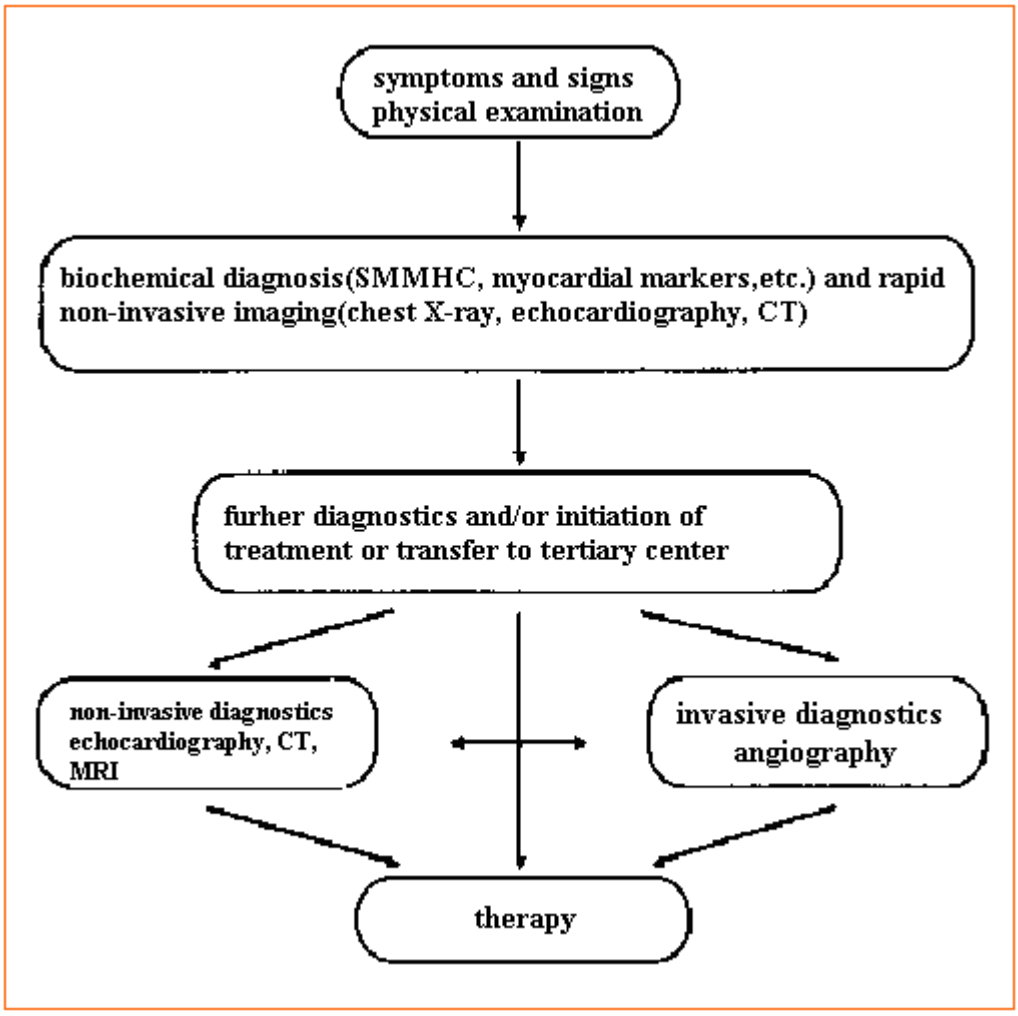


Figure 8. Diagnostic algorithm of AD incorporating biochemical diagnosis (61).

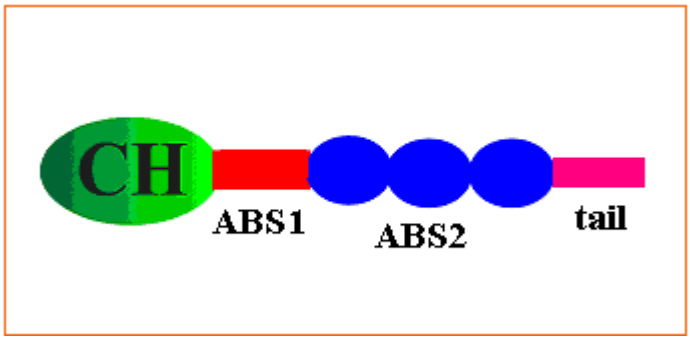
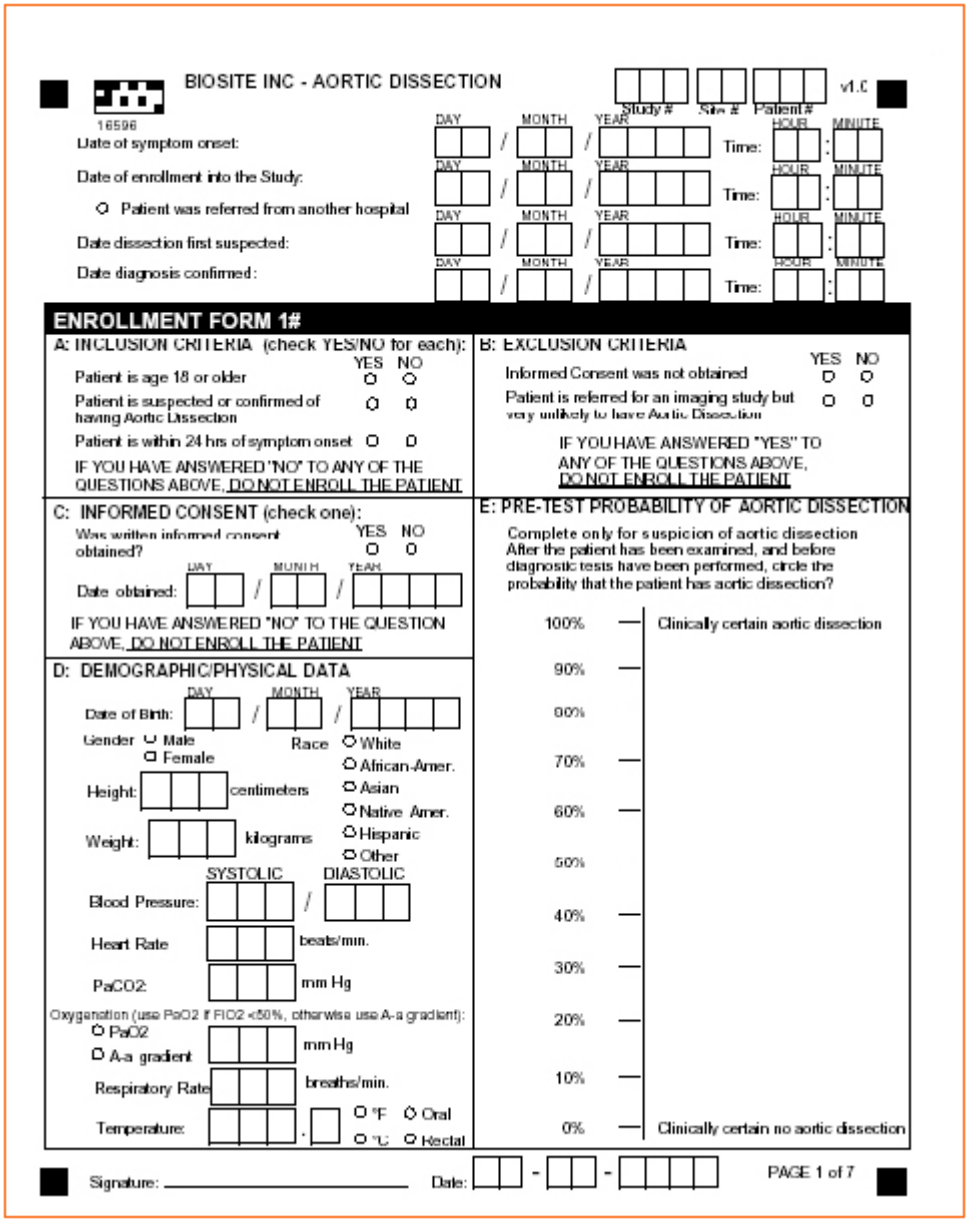


Figure 9. The delineated functional and structural domains in calponins.


BIOSITE INC - AORTIC DISSECTION

Study # Site # Patient # v1.0

16596

Date of symptom onset: DAY / MONTH / YEAR Time: HOUR : MINUTE

Date of enrollment into the Study: DAY / MONTH / YEAR Time: HOUR : MINUTE

○ Patient was referred from another hospital

Date dissection first suspected: DAY / MONTH / YEAR Time: HOUR : MINUTE

Date diagnosis confirmed: DAY / MONTH / YEAR Time: HOUR : MINUTE

ENROLLMENT FORM 1#

A: INCLUSION CRITERIA (check YES/NO for each):

	YES	NO
Patient is age 18 or older	○	○
Patient is suspected or confirmed of having Aortic Dissection	○	○
Patient is within 24 hrs of symptom onset	○	○

IF YOU HAVE ANSWERED "NO" TO ANY OF THE QUESTIONS ABOVE, DO NOT ENROLL THE PATIENT

B: EXCLUSION CRITERIA

	YES	NO
Informed Consent was not obtained	○	○
Patient is referred for an imaging study but very unlikely to have Aortic Dissection	○	○

IF YOU HAVE ANSWERED "YES" TO ANY OF THE QUESTIONS ABOVE, DO NOT ENROLL THE PATIENT

C: INFORMED CONSENT (check one):

Was written informed consent obtained? YES ○ NO ○

Date obtained: DAY / MONTH / YEAR

IF YOU HAVE ANSWERED "NO" TO THE QUESTION ABOVE, DO NOT ENROLL THE PATIENT

D: DEMOGRAPHIC/PHYSICAL DATA

Date of Birth: DAY / MONTH / YEAR

Gender ○ Male ○ Female

Race ○ White ○ African-Amer. ○ Asian ○ Native Amer. ○ Hispanic ○ Other

Height: centimeters

Weight: kilograms

Blood Pressure: SYSTOLIC / DIASTOLIC

Heart Rate: beats/min.

PaCO2: mm Hg

Oxygenation (use PaO2 if FIO2 <50%, otherwise use A-a gradient):

○ PaO2 ○ A-a gradient: mm Hg

Respiratory Rate: breaths/min.

Temperature: . ○ °F ○ °C ○ Oral ○ Rectal

E: PRE-TEST PROBABILITY OF AORTIC DISSECTION

Complete only for suspicion of aortic dissection. After the patient has been examined, and before diagnostic tests have been performed, circle the probability that the patient has aortic dissection?

100% — Clinically certain aortic dissection

90%

80%

70%

60%

50%

40%

30%

20%

10%

0% — Clinically certain no aortic dissection

Signature: _____ Date: - -

PAGE 1 of 7

Figure 10. The first page of Case Report Form (CRF).

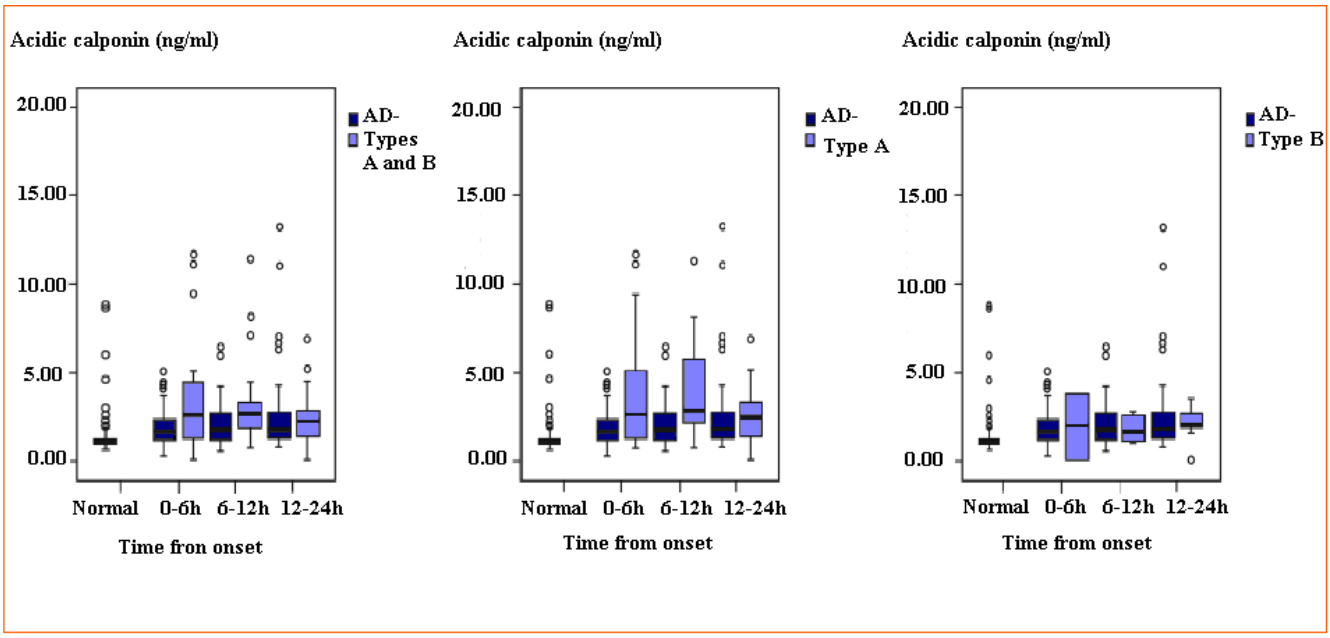


Figure 11. Box plots of acidic calponin in patients with aortic dissection according to type (all, type A, type B) and time after symptom onset (0–6, 6–12, 12–24 h).

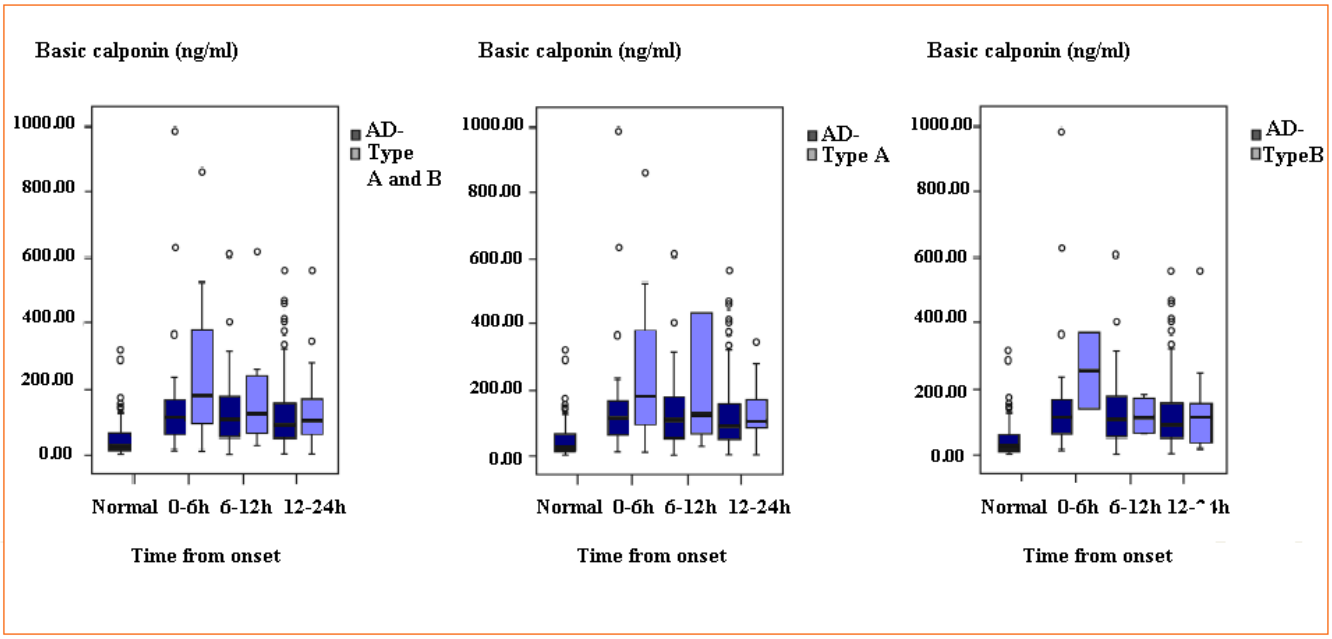


Figure 12. Box plots of basic calponin in patients with aortic dissection according to type (all, type A, type B) and time after symptom onset (0–6, 6–12, 12–24 h).

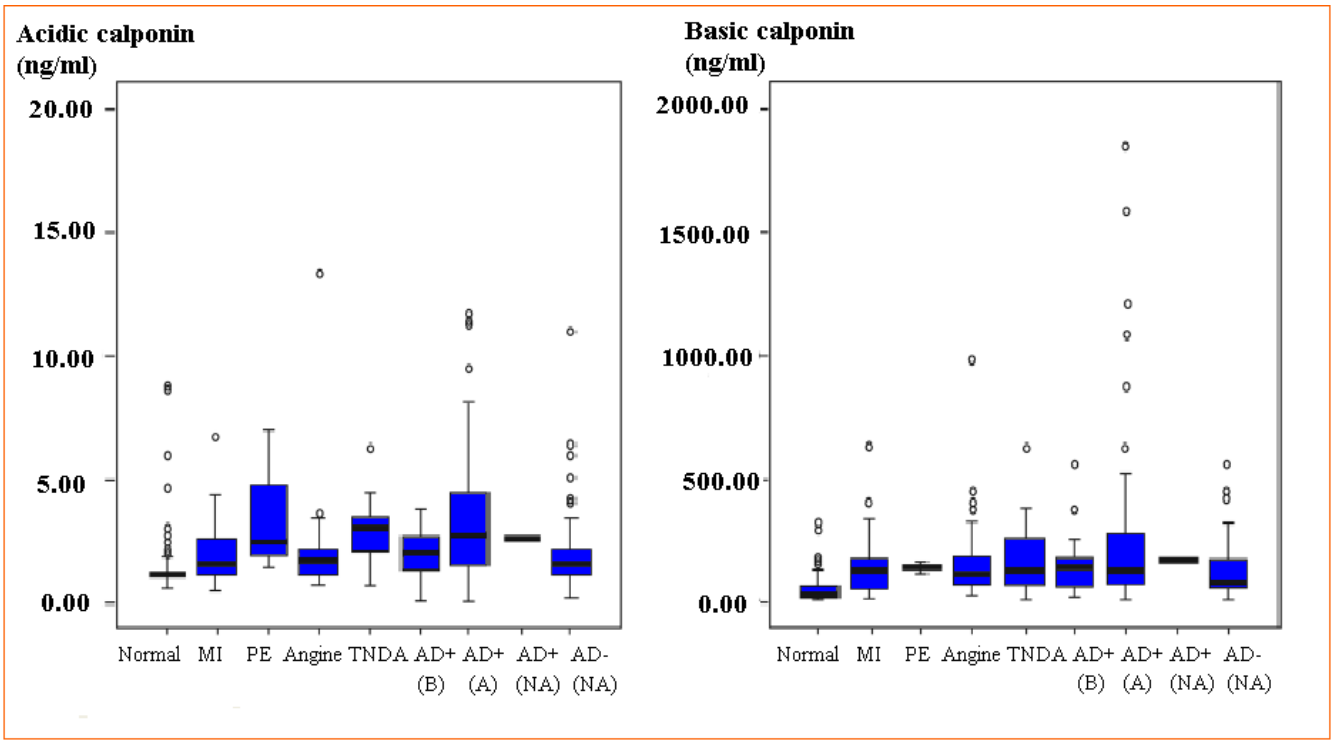


Figure 13. Acidic and basic calponin levels in patients examined in the present study according to final diagnosis. MI, myocardial infarction; PE, pulmonary embolism; TNDA, thoracic non-dissecting aneurysm; AD+(B), type B aortic dissection; AD+(A), type A aortic dissection; AD +, aortic dissection type not determined; AD -, uncertain final diagnosis but not aortic dissection.

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